

Cable, Jemma (2012) An investigation into the role and effects of the endocannabinoid system in adipocytes.
PhD thesis, University of Nottingham.

Access from the University of Nottingham repository:

http://eprints.nottingham.ac.uk/12708/1/Jemma_Cable_PhD_Thesis.pdf

Copyright and reuse:

The Nottingham ePrints service makes this work by researchers of the University of Nottingham available open access under the following conditions.

- Copyright and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners.
- To the extent reasonable and practicable the material made available in Nottingham ePrints has been checked for eligibility before being made available.
- Copies of full items can be used for personal research or study, educational, or not-for-profit purposes without prior permission or charge provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.
- Quotations or similar reproductions must be sufficiently acknowledged.

Please see our full end user licence at:

http://eprints.nottingham.ac.uk/end_user_agreement.pdf

A note on versions:

The version presented here may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the repository url above for details on accessing the published version and note that access may require a subscription.

For more information, please contact eprints@nottingham.ac.uk

**An investigation into the role and effects of the
endocannabinoid system in adipocytes**

Jemma Cable, BSc.

**Thesis submitted to the University of
Nottingham for the degree of Doctor of
Philosophy**

September 2011

Abstract

In recent years evidence has emerged that the endocannabinoid system (ECS) may have a significant role in metabolism and energy homeostasis. Several studies have identified upregulation of the peripheral ECS in obesity and type 2 diabetes, but the mechanisms behind this and the consequences of upregulation are unclear. The aim of this thesis was to further elucidate the role of the ECS in mature adipocytes, and its activity in obesity and related metabolic dysfunction.

Three adipose tissue depots were dissected from lean, obese and obese diabetic Zucker rats ($n=6-8$). In human studies, written informed consent was obtained from healthy volunteers within the University of Nottingham and obese surgical patients at the Royal Derby Hospital. Anthropometric measurements and venous blood samples were obtained. In these studies, subcutaneous abdominal adipose tissue was taken from all subjects ($n=28$ healthy study; $n=27$ surgical study), and visceral adipose tissue was obtained from some of the surgical patients ($n=14$). In all studies, collagenase was used to isolate mature adipocytes from the adipose tissue, and FAAH and MGL activities in the adipocytes were assayed using tritium labelled substrates. Human subcutaneous preadipocytes (Promocell, Germany) were cultured and differentiated. Adipocytes were cultured with high concentrations of glucose (15 mM) and/or insulin (1 μ M) for 24 hours, in combination with anandamide or 2-AG for 2 or 24 hours. Adiponectin, leptin and resistin in the cell culture media were then measured using sandwich ELISAs. In another study, anandamide and 2-AG uptake were measured in differentiated adipocytes after 2 or 24 hours' stimulation with glucose and/or insulin. FAAH and MGL activities in the cultured adipocytes were also measured in this study.

In rats, FAAH and MGL activities correlated with body mass. In healthy humans, FAAH activity in subcutaneous adipocytes correlated with BMI and waist circumference, but not with other anthropometric measurements, serum glycaemic markers or adipokines. In obese patients, the enzyme activities had no relationships with any of the anthropometric or metabolic markers investigated. Furthermore, there were no differences in activity between patients with metabolic syndrome or diabetes and those without. In both rats and humans, there were no significant differences in FAAH and MGL activities between subcutaneous and visceral adipocytes. In the cell culture studies, anandamide and 2-AG did not alter adipokine secretion under normal, high glucose or high insulin conditions. Chronic insulin exposure increased anandamide uptake, but none of the other acute or chronic treatments with glucose and/or insulin affected anandamide or 2-AG uptake. Glucose and insulin were found to reduce MGL activity.

These studies suggest that the rate of anandamide hydrolysis in mature adipocytes is increased in obesity. This relationship was not apparent in a morbidly obese sample. MGL activity in humans does not have relationships with adiposity or metabolic markers, and this may reflect its role as a major component of lipid metabolism, particularly lipolysis. Anandamide and 2-AG are unlikely to be direct mediators of adipokine secretion, at least in cell culture. Insulin may affect endocannabinoid signalling in adipocytes by increasing anandamide uptake and suppressing MGL activity. Overall, these results support the notion that the ECS in adipocytes is dysregulated in obesity, but this is not driven by specific factors associated with obesity.

Publications and presentations arising from this thesis

Peer reviewed article

Cable JC, Tan GD, Alexander SP, O'Sullivan SE (2011). The activity of the endocannabinoid metabolising enzyme fatty acid amide hydrolase in subcutaneous adipocytes correlates with BMI in metabolically healthy humans. *Lipids Health Dis.* 10:129.

Published abstracts and presentations

Cable JC, Tan GD, Alexander SP, O'Sullivan SE (2010). Fatty acid amide hydrolase and monoacylglycerol lipase activities in adipocytes taken from obese surgical patients. British Pharmacological Society Meeting 2010, London, UK. Proceedings of the British Pharmacological Society at <http://www.pA2online.org/abstracts/Vol8Issue1abst148P.pdf>.

Oral communication.

Cable JC, Tan GD, Alexander SP, O'Sullivan SE (2010). Catabolic enzymes of the endocannabinoid system in adipocytes from metabolically healthy humans, British Pharmacological Society Meeting 2010, London, UK. Proceedings of the British Pharmacological Society at <http://www.pA2online.org/abstracts/Vol8Issue1abst145P.pdf>.

Oral communication.

Cable JC, Tan GD, Alexander SP, O'Sullivan SE (2010). Fatty acid amide hydrolase activity in human adipocytes does not correlate with metabolic markers or anthropometric measurements, 20th Annual Symposium of the International Cannabinoid Research Society. Poster presentation.

Cable JC, O'Sullivan SE, Fowler, CJ (2010). Insulin increases anandamide uptake in cultured human adipocytes, 20th Annual Symposium of the International Cannabinoid Research Society. Poster presentation.

Cable JC, Alexander SP, Tan GD, O'Sullivan SE (2009). A comparative study of fatty acid amide hydrolase activity between three adipose depots in Zucker obese and Zucker diabetic rats. British Pharmacological Society Meeting 2009, London, UK. Proceedings of the British Pharmacological Society at <http://www.pA2online.org/abstracts/Vol7Issue4abst020P.pdf>.
Oral communication.

Acknowledgements

My most sincere thanks go to Saoirse O'Sullivan, who has been the best mentor I could have hoped for. She taught and guided me with remarkable patience and understanding, and has been a truly impressive role model, both professionally and personally. I am also absolutely indebted to Garry Tan. I left every meeting I had with him feeling more confident and positive than when I entered! I would also like to thank Steve Alexander for sharing his expert knowledge in the field of enzyme assays, cannabinoids, lipids and life with me. Margaret Baker was invaluable during the taking of samples in this project, and I would like to thank her for this, and for regularly bringing in home-made cakes.

I completed some of the work in this project in Sweden under the expert and friendly guidance of Chris Fowler, who was incredibly supportive and generous. I would like to thank him and his team, especially Lina and Eva, for all their time and help, and for making me so welcome.

Much of the work presented herein could not have been done without the donation of samples from many people, and the co-operation of surgeons, particularly Mr Javed Ahmed and Prof Mike Larvin. My sincere thanks go to all of these people.

Everyone in the laboratories at Derby and QMC has been brilliant, and made the past three years highly enjoyable. I would particularly like to thank Chris S and Willy one-time for their banter and reading most of this thesis; Tayyba and Abdul for being such calming influences; and Susann, Chris T, Dave, Natasha, Vince and Marie for between them managing to be highly entertaining and, at times, actually quite helpful.

I have had unwavering and unconditional love and support from Mum, Dad, Maddy and Kirsty at all times, and I can't thank you enough. Above all, I would like to thank you for reminding me at every suitable opportunity that I'm really not all that clever.

Most importantly, Andrew. I wouldn't have got to this point without you.

Contents

Abstract	1
Publications and presentations arising from this thesis	3
Peer reviewed article.....	3
Published abstracts and presentations	3
Acknowledgements	5
Contents	7
List of Figures.....	12
List of Tables	13
Abbreviations	14
1. Introduction	15
1.1.1 The Endocannabinoids	15
1.1.2 Endocannabinoid synthesis pathways.....	16
1.1.2.1 Anandamide synthesis	17
1.1.2.2 2-AG synthesis.....	17
1.1.3 Endocannabinoid degradation pathways	19
1.1.3.1 Fatty acid amide hydrolase	21
1.1.3.2 Monoacylglycerol lipase.....	22
1.1.4 Cannabinoid receptors	23
1.2 Adipose tissue.....	26
1.2.1 Lipid metabolism.....	27
1.2.2 The endocrine function of adipose tissue: adipokines	28
1.2.2.1 Adiponectin.....	29
1.2.2.2 Leptin	30
1.2.2.3 Resistin.....	31
1.3 Obesity.....	33
1.3.1 Obesity	33
1.3.2 Type 2 diabetes	33
1.3.3 Dyslipidaemia in obesity.....	34
1.4 The ECS in adipocytes and adipose tissue	35
1.4.1 Endocannabinoids	35
1.4.2 Receptors	35
1.4.3 Enzymes.....	37
1.5 The ECS in the CNS and adipose tissue in obesity and diabetes.....	38
1.5.1 CNS.....	38
1.5.2 Rimonabant and the CB ₁ receptor in metabolism and obesity ...	39
1.5.3 Cannabinoid receptor expression in obesity.....	42
1.5.4 Endocannabinoid levels in obesity	44

1.5.5 FAAH and MGL in obesity	45
1.6 The role of the ECS in metabolic homeostasis and obesity in other organs.....	47
1.6.1 Pancreas	47
1.6.2 Skeletal muscle.....	48
1.6.3 Liver.....	49
1.7 Summary	49
1.8 Aims.....	51
2. Materials and Methods	53
2.1 Zucker rats.....	53
2.1.1 Rat models of obesity.....	53
2.1.2 Animal housing and food	53
2.1.3 Adipose tissue collection	53
2.1.4 Blood glucose measurement	54
2.2 Adipocyte isolation and preparation	54
2.2.1 Purification of mature adipocytes.....	55
2.2.2 Homogenisation of mature adipocytes	55
2.3 Healthy human volunteers	56
2.3.1 Ethics and screening	56
2.3.2 Anthropometric measurements	56
2.3.3 Adipose tissue collection	58
2.3.4 Blood collection	59
2.4 Surgical patients	59
2.4.1 Ethics for surgical study	59
2.4.2 Anthropometric measurements	59
2.4.3 Adipose tissue collection	59
2.5 Enzyme activity assays	60
2.5.1 Fatty acid amide hydrolase (FAAH) activity assay	60
2.5.2 Monoacylglycerol lipase (MGL) activity assay	61
2.5.3 Enzyme activity optimisation	62
2.5.4 Protein assay	63
2.6 Cannabinoid receptor assays.....	64
2.6.1 GTPyS binding assay	64
2.6.2 CB ₁ receptor ELISA	64
2.7 Biochemical assays.....	65
2.7.1 Glucose assay	65
2.7.2 Insulin assay	65
2.7.3 Adiponectin, leptin and resistin	66

2.8 Cell culture experiments	67
2.8.1 Adipocyte culture	67
2.8.1.1 Oil Red O staining of adipocytes	68
2.8.2 Effects of endocannabinoids on adipokine secretion	70
2.8.3 Endocannabinoid uptake assay	70
2.9 Chemicals, solutions and buffers	72
2.10 Statistical analysis	73
2.10.1 Enzyme activity assays.....	73
2.10.2 Adipocyte culture adipokine secretion	74
2.10.3 Endocannabinoid uptake	74
3. A study of the ECS in mature adipocytes from lean, obese and diabetic Zucker rats	75
3.1 Introduction	75
3.2 Aims	78
3.3 Materials and Methods	78
3.3.1 Zucker rats and adipocyte preparation	78
3.3.2 GTPyS binding assay	79
3.3.3 FAAH and MGL assays.....	79
3.3.4 Protein assay.....	79
3.3.5 Statistical analysis.....	79
3.4 Results	80
3.4.1 Characteristics of Zucker rats.....	80
3.4.2 Cannabinoid receptor assays.....	80
3.4.3 Enzyme activity	82
3.4.4 Enzyme activity in three rat strains	83
3.4.5 FAAH activity and body mass	85
3.4.6 MGL activity and body mass.....	85
3.4.7 Enzyme activity between adipose tissue depots	87
3.4.8 Enzyme activity and blood glucose	89
3.5 Discussion.....	91
4. Enzyme activities in subcutaneous human mature adipocytes	100
4.1 Introduction	100
4.2 Aims	102
4.3 Materials and Methods	102
4.3.1 Subjects	102
4.3.2 Homogenisation of adipocytes	103
4.3.3 FAAH and MGL activity assay	103
4.3.4 Protein assays	103

4.3.5 Blood serum assays	103
4.3.5 Statistical analysis	103
4.4 Results.....	104
4.4.1 Subject characteristics.....	104
4.4.2 Enzyme activity, BMI, body fat and circumferences.....	106
4.4.4 Enzyme activity and blood pressure	107
4.4.5 Enzyme activity and insulin sensitivity	107
4.5 Discussion	109
5. FAAH and MGL in mature adipocytes from obese patients	113
5.1 Introduction	113
5.2 Aims.....	114
5.3 Materials and Methods	114
5.3.1 Patient recruitment	114
5.3.2 Healthy, metabolic syndrome and diabetic grouping	115
5.3.3 Medications	115
5.3.4 Adipose tissue sample collection.....	116
5.3.5 Adipocyte isolation and enzyme assays.....	116
5.4 Results.....	116
5.4.1 Patient characteristics	116
5.4.2 Enzyme activities, BMI and waist circumference	117
5.4.3 Enzyme activities and HbA1c	118
5.4.4 Enzyme activities in different metabolic groups	119
5.4.5 Enzymes in subcutaneous and visceral adipocytes	121
5.5 Discussion	122
6. The effects of endocannabinoids on adipokine secretion in cultured human adipocytes	128
6.1 Introduction	128
6.2 Aims.....	130
6.3 Materials and Methods	130
6.3.1 Adipocyte culture.....	130
6.3.2 Insulin and glucose	130
6.3.3 Anandamide and 2-AG.....	131
6.3.4 Adipokine assays	131
6.3.5 Statistical analysis	131
6.4 Results.....	132
6.4.1 Effects of glucose and insulin	132
6.4.2 Acute endocannabinoid treatment and adiponectin	132
6.4.3 Acute endocannabinoid treatment and leptin	134

6.4.4 Chronic endocannabinoid treatment and adiponectin	135
6.4.5 Chronic endocannabinoid treatment and leptin	137
6.4.6 Resistin in cultured adipocytes.....	139
6.5 Discussion.....	140
7. Endocannabinoid uptake in cultured human adipocytes	143
7.1 Introduction	143
7.2 Aims	145
7.3 Materials and Methods	146
7.3.1 Endocannabinoid uptake experiments	146
7.3.2 FAAH and MGL activity experiments	146
7.3.3 Statistical analysis.....	146
7.4 Results	147
7.4.1 Anandamide uptake.....	147
7.4.2 FAAH inhibition and anandamide uptake.....	147
7.4.3 2-AG uptake.....	148
7.4.4 MGL inhibition and 2-AG uptake.....	148
7.4.5 Enzyme activity	149
7.5 Discussion.....	151
8. General discussion	155
8.1 Limitations and future work.....	159
8.2 Conclusion	161
References	162

List of Figures

Figure 1.1 Chemical structures of the endocannabinoids.	16
Figure 1.2 Endocannabinoid synthesis pathways	18
Figure 1.3 Degradation pathways for anandamide	20
Figure 1.4 Degradation pathways for 2-AG	21
Figure 1.5 Lipid uptake by adipocytes.....	27
Figure 1.6 Lipolysis	28
Figure 2.1 Adipose tissue dissection	54
Figure 2.2 Anthropometric measurement sites	57
Figure 2.3 FAAH assay	60
Figure 2.4 Optimisation of FAAH and MGL assays.....	63
Figure 2.5 Differentiated human subcutaneous adipocytes in culture.	69
Figure 3.1 GTPyS binding	81
Figure 3.2 Inhibition of FAAH and MGL	82
Figure 3.3 FAAH and MGL in three rat strains	84
Figure 3.4 FAAH and MGL with body mass	86
Figure 3.5 FAAH and MGL in three adipose tissue depots	88
Figure 3.6 FAAH and MGL with blood glucose	89
Figure 4.1 Correlations between BMI and other variables	105
Figure 4.2 FAAH and MGL with body fat estimates	106
Figure 4.3 FAAH and MGL with waist circumference.....	107
Figure 4.4 FAAH and MGL with blood pressure.....	107
Figure 4.5 FAAH and MGL with insulin sensitivity	108
Figure 5.1 FAAH and MGL with BMI and waist circumference.....	118
Figure 5.2 FAAH and MGL with HbA1c	119
Figure 5.3 FAAH and MGL in three metabolic groups.....	120
Figure 5.4 FAAH and MGL in subcutaneous and visceral adipocytes	121
Figure 6.1 Effects of acute anandamide on adiponectin secretion	132
Figure 6.2 Effects of acute 2-AG on adiponectin secretion.....	133
Figure 6.3 Effects of acute anandamide on leptin secretion	134
Figure 6.4 Effects of acute 2-AG on leptin secretion.....	135
Figure 6.5 Effects of chronic anandamide on adiponectin secretion.....	136
Figure 6.6 Effects of chronic 2-AG on adiponectin secretion	137

Figure 6.7 Effects of chronic anandamide on leptin secretion	138
Figure 6.8 Effects of chronic 2-AG on leptin secretion.....	139
Figure 7.1 Anandamide uptake	148
Figure 7.2 2-AG uptake	149
Figure 7.3 MGL activity.....	150

List of Tables

Table 1.1 Cannabinoids and adipocytes	37
Table 2.1 Body density formulae	58
Table 4.1 Subject characteristics.....	105
Table 5.1 Characteristics of patients	117

Abbreviations

2-AG	2-arachidonoylglycerol
ATGL	Adipose triglyceride lipase
AEA	Anandamide, N-arachidonoyl ethanolamide
BMI	Body mass index
CB receptor	Cannabinoid receptor
CNS	Central nervous system
COX	Cyclooxygenase
DAGL	Diacylglycerol lipase
DMSO	Dimethyl sulfoxide
DPM	Degradations per minute
ECS	Endocannabinoid system
ELISA	Enzyme-linked immunosorbent assay
FAAH	Fatty acid amide hydrolase
FABP	Fatty acid binding protein
GPCR	G protein-coupled receptor
HDL	High density lipoprotein
HSL	Hormone sensitive lipase
KRH	Krebs-Ringer-Hepes
LOX	Lipoxygenases
LPL	Lipoprotein lipase
MGL	Monoacylglycerol lipase
mRNA	Messenger ribonucleic acid
NAE	N-acyl ethanolamine
NAPE	N-arachidonylphosphatidylethanolamine
NADA	Arachidonoyl dopamine
OEA	Oleoylethanolamine
PEA	Palmitoylethanolamide
PLD	Phospholipase D
PPAR	Peroxisome proliferator-activated receptor
RXR	Retinoid X receptor
THC	Δ^9 -tetrahydrocannabinol
TMB	Tetramethylbenzidine
TRP	Transient receptor potential
TRPV	Transient receptor potential vanilloid
VLDL	Very low density lipoprotein
ZDF	Zucker diabetic fatty rats

1. Introduction

The endocannabinoid system (ECS) is a complex and ubiquitously expressed signalling system that comprises the endocannabinoids, their receptors and the enzymes required for endocannabinoid synthesis and degradation. The ECS has been identified in most human organs and tissues, and the system has roles in a wide range of normal and pathological processes, such as pain, energy homeostasis, fertility, immune responses and behaviour, with both central and peripheral involvement (for review see De Petrocellis and Di Marzo, 2009). Initially interest in the ECS was focussed on its presence and role in the central nervous system (CNS), but more recently its role and regulation in adipose tissue and metabolism has been a matter of intense research. Despite this work and many significant advances in knowledge, in many cases, the molecular mechanisms and physiological significance of the ECS have yet to be fully elucidated.

1.1.1 The Endocannabinoids

The endocannabinoids identified to date are mostly lipid derivatives of arachidonic acid. The two most extensively characterised endocannabinoids are *N*-arachidonylethanolamide (anandamide), which was the first to be described (Devane *et al.*, 1992), and its glycerol ester analogue 2-arachidonoylglycerol (2-AG) which was reported a few years later (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). The degradation of these two endocannabinoids is one of the major foci of this thesis. There are many other compounds which are sometimes referred to as endocannabinoids, due to their activity at one or more of the cannabinoid receptors (for extensive review on cannabinoid pharmacology see Pertwee *et al.*, 2010). Three such molecules are virodhamine (Porter *et al.*, 2002), 2-arachidonoyl glyceryl ether (noladin ether) (Hanus *et al.*, 2001) and *N*-arachidonoyl dopamine (NADA) (Bisogno *et al.*, 2000). Virodhamine has a very similar structure to anandamide, but opposite orientation around the arachidonic acid-ethanolamine bond (Porter *et al.*, 2002). Likewise, noladin ether and 2-AG are structurally similar (see Figure 1.1).

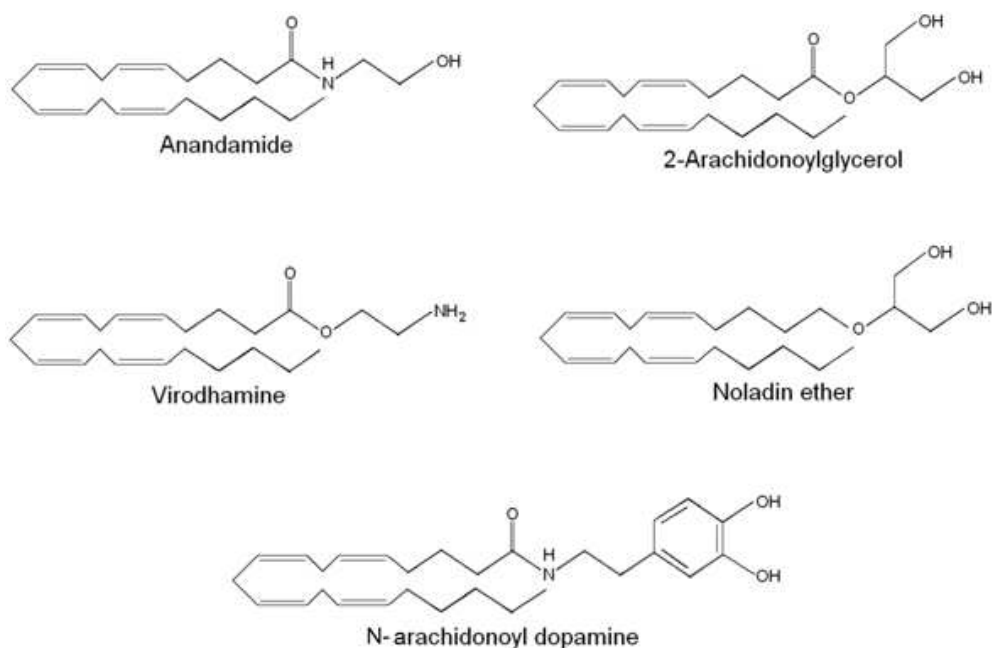


Figure 1.1 Chemical structures of the endocannabinoids.

In addition to these, there are other *N*-acylethanolamines (NAEs), similar in structure to anandamide, which are not usually considered to be endocannabinoids, but which are ligands for some of the same non-cannabinoid receptor targets as endocannabinoids. Two of these, oleoylethanolamine (OEA) and palmitoylethanolamide (PEA) have been extensively investigated as they are present at higher concentrations than the endocannabinoids in many mammalian tissues and are metabolised by the same enzymes (Alexander and Kendall, 2007; Lambert and Muccioli, 2007).

In addition to the endocannabinoids, there are many other cannabinoid receptor ligands, from plant and synthetic sources. Two of the most common phytocannabinoids (from *Cannabis sativa*) are Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD). THC was one of the first cannabinoids to be identified (Gaoni and Mechoulam, 1964) and there are at least 60 further phytocannabinoids with wide ranging pharmacology (for reviews see Pertwee and Ross, 2002; Elsohly and Slade, 2005).

1.1.2 Endocannabinoid synthesis pathways

Anandamide and 2-AG are thought to be synthesised predominantly via cleavage of membrane phosphoglyceride precursors (as reviewed by Bisogno, 2008). As the endocannabinoid precursors are a normal component of cell membranes, it is the presence or absence of endocannabinoid synthesising enzymes and receptors that dictate whether

the ECS will be activated and where it will act (for review see Di Marzo *et al.*, 2007). Endocannabinoids are often referred to as signalling molecules which are synthesised “on demand” and, in neurons at least, endocannabinoids are synthesised and released very rapidly upon stimulation (for review see Alger and Kim, 2011). There is however mounting evidence that under certain conditions endocannabinoids may be stored before their release (for reviews see Maccarrone *et al.*, 2010; Alger and Kim, 2011). Several anabolic pathways for anandamide and 2-AG have been described to date, and alternative enzymes are currently under investigation, suggesting that endocannabinoid regulation has the potential to be very complex.

1.1.2.1 Anandamide synthesis

The major pathway for anandamide synthesis involves an *N*-acyltransferase to convert 1,2-di-arachidonylphosphatidylcholine to *N*-arachidonylphosphatidylethanolamine (NAPE) (Astarita *et al.*, 2008). Hydrolysis of this molecule by a Ca^{2+} -sensitive NAPE-selective phospholipase D (NAPE-PLD) then yields anandamide (Okamoto *et al.*, 2004; Wang *et al.*, 2008a).

The presence of anandamide in NAPE-PLD knockout mice (Leung *et al.*, 2006) indicates that alternative pathways exist to convert phosphatidylethanolamine to anandamide. One of the proposed pathways relies on a phospholipase C and protein tyrosine phosphatase N22 (Liu *et al.*, 2006), while another uses a phospholipase A_2 and a lyso-phospholipase D (Sun *et al.*, 2004) (see Figure 1.2a). The physiological relevance of these alternative pathways under normal conditions in humans has yet to be explored (reviewed by Muccioli, 2010).

1.1.2.2 2-AG synthesis

In common with anandamide, the biosynthesis of 2-AG has been shown to be possible via more than one pathway. The pathway thought to be physiologically important in most cells and under normal conditions is the conversion of phosphatidylinositol, via a phospholipase C, to diacylglycerol, which is subsequently hydrolysed by a non-specific diacylglycerol lipase (DAGL) to 2-AG (Kondo *et al.*, 1998; Bisogno *et al.*, 2003). The intermediate in this pathway, diacylglycerol, can also be generated from phosphatidic acid under the action of a phosphatidic acid hydrolase (Bisogno *et al.*, 1999). Alternatively, 2-AG may be synthesised from a

lysophospholipid under the action of a lyso-phospholipase C (Ueda *et al.*, 1993; Sugiura *et al.*, 1995) (see Figure 1.2b). It is also likely that, in adipocytes at least, a proportion of 2-AG synthesis occurs as a result of triglyceride lipolysis through adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) (Zechner *et al.*, 2009).

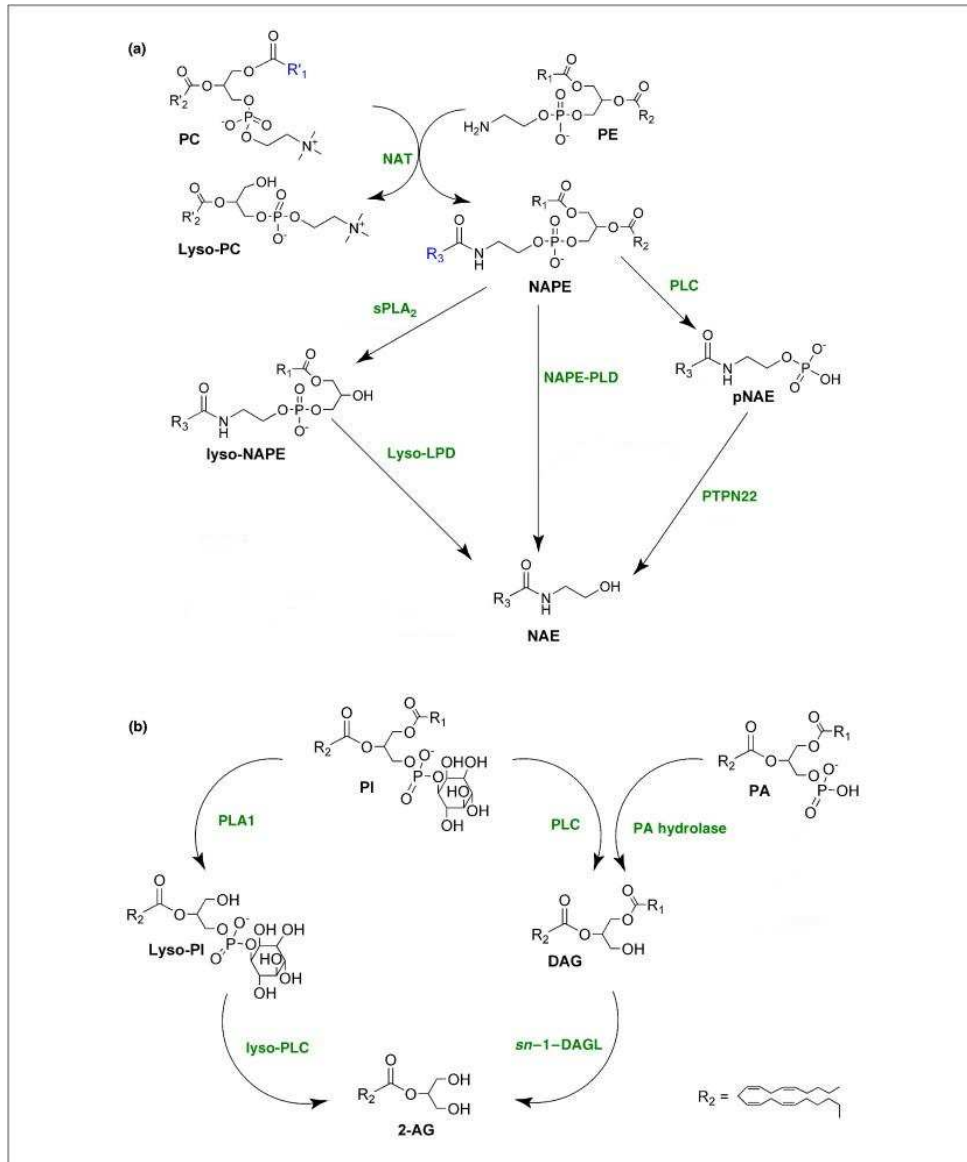


Figure 1.2 Endocannabinoid synthesis pathways

Endocannabinoid synthesis pathways for anandamide (a) and 2-AG (b) (adapted from Muccioli, 2010). PC, phosphatidylcholine; DAG, diacylglycerol; pNAE, phospho-NAE; lyso-NAPE, lysophosphatidyl-NAPE; lyso-PLC, lysophosphatidylinositol-selective phospholipase C; NAPE, N-acylphosphatidylethanolamine; NAPE-PLD, N-acylphosphatidylethanolamine-selective phospholipase D; NAT, N-acyltransferase; PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLA₁, phospholipase A₁; PLC, phospholipase C; PTPN22, phosphatase protein tyrosine phosphatase 22; sPLA₂, secreted phospholipase A₂.

1.1.3 Endocannabinoid degradation pathways

Endocannabinoid signalling is terminated in two stages. Cellular uptake of the endocannabinoids occurs first, via mechanisms that are still under debate (reviewed by Yates and Barker, 2009b), and this is discussed in detail in Chapter 7. Then intracellular enzymes catalyse endocannabinoid degradation. The major pathways for anandamide and 2-AG catabolism are well characterised, but as with the synthesis pathways, alternative catabolic pathways are likely to exist.

Anandamide is predominantly degraded under the action of fatty acid amide hydrolase (FAAH) (Deutsch and Chin, 1993; Cravatt *et al.*, 1996). Indeed, the rate of anandamide hydrolysis in tissues from FAAH knockout mice is 50-100 fold reduced, and anandamide concentrations in the brain (Cravatt *et al.*, 2001; Lichtman *et al.*, 2002) and liver (Tourino *et al.*, 2010) are elevated.

2-AG is inactivated by monoacylglycerol lipase (MGL) and also by FAAH to some extent (Dinh *et al.*, 2002b), although anandamide is hydrolysed at a faster rate and more efficiently than 2-AG by FAAH (Di Marzo *et al.*, 1998). However, the rate of anandamide hydrolysis by FAAH is reduced when the concentration of 2-AG is high, as is the case in many cells (Di Marzo *et al.*, 1998). In animal studies it has been shown that, despite the ability of FAAH to metabolise 2-AG, MGL appears to have the pivotal role. In homogenised mouse brain, MGL accounts for approximately 85% of 2-AG hydrolysis, with FAAH contributing just 1% (Blankman *et al.*, 2007). In mice, intraperitoneal administration of JZL184 (a specific MGL inhibitor) causes an increase in brain 2-AG concentrations (Long *et al.*, 2009). Similarly, in rats, depletion of MGL causes a significant decrease in the rate of 2-AG hydrolysis (Dinh *et al.*, 2004).

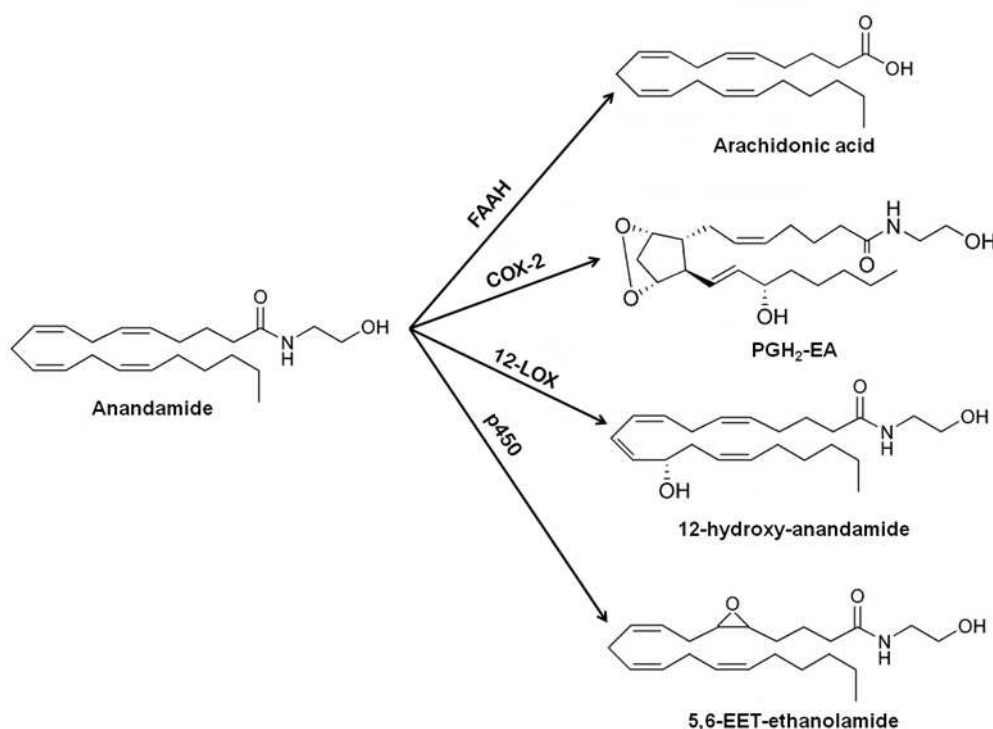


Figure 1.3 Degradation pathways for anandamide

PGH₂-EA, prostaglandin H₂ ethanolamide; EET, epoxyeicosatrienoic acid (adapted from Muccioli, 2010).

As an alternative to hydrolysis, anandamide may be oxidised by cyclooxygenase-2 (COX-2) to PGE₂-ethanolamide (Ross *et al.*, 2002), which is a prostaglandin based molecule. The 12- and 15-lipoxygenases (12- and 15-LOX) can also use anandamide as a substrate to yield 12- and 15-hydroxy-anandamide (Ueda *et al.*, 1995b). Alternatively, the family of cytochrome p450 enzymes can catalyse the conversion of anandamide to various polar lipids (for review see Burstein *et al.*, 2000) (see Figure 1.3). Similarly, 2-AG has been shown to be a substrate for these enzymes, with COX-2 yielding prostaglandin H₂ glycerol ester (Kozak *et al.*, 2000), 12-LOX giving 12(S)-hydroperoxyeicosa-5,8,10,14-tetraenoic acid (Moody *et al.*, 2001) and 15-LOX giving 15(S)-hydroperoxyeicosatetraenoic acid glyceryl ester (Kozak *et al.*, 2002) (Figure 1.4). It should be noted that many of these studies have been conducted using animal tissues and FAAH or MGL inhibitors, so the physiological prevalence and relevance of these alternative endocannabinoid inactivation pathways has yet to be demonstrated in humans (for review see Fowler, 2007).

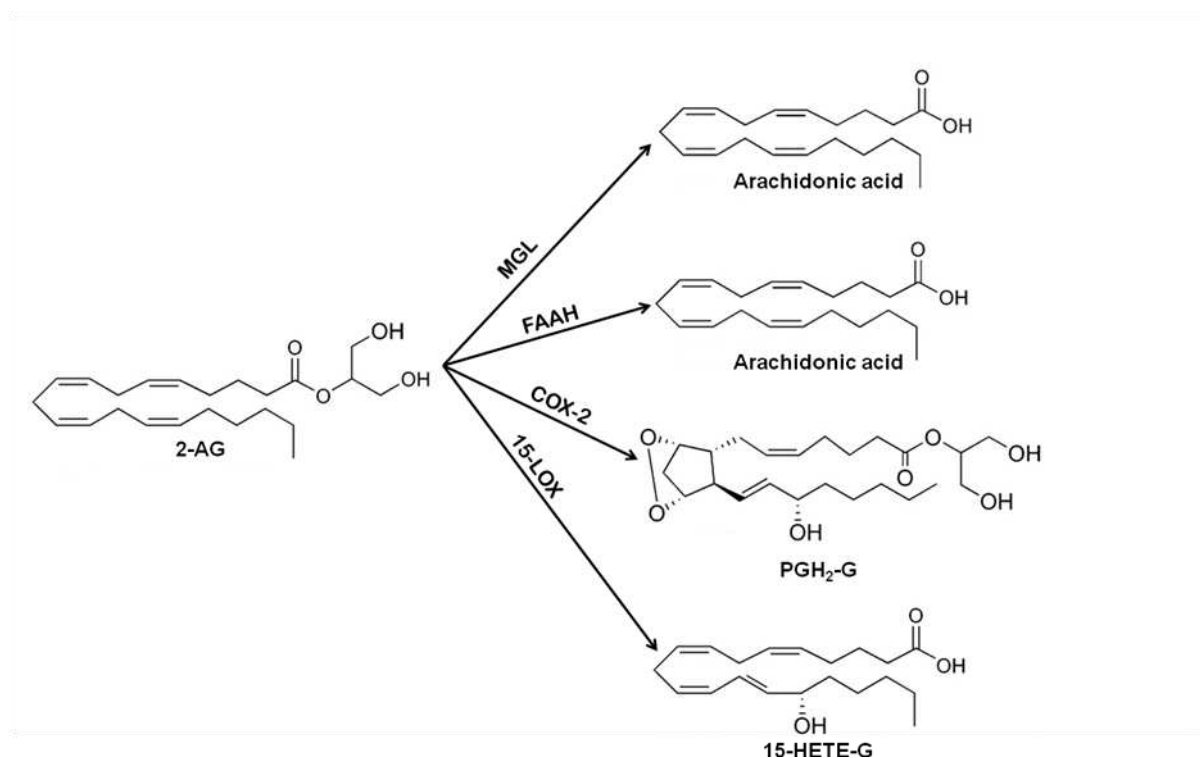


Figure 1.4 Degradation pathways for 2-AG

15-HETE-G, 15-hydroxyeicosatetraenoic acid glyceryl ester (from Muccioli, 2010).

Given that FAAH and MGL are thought to contribute the majority of endocannabinoid inactivation *in vivo*, the activities of these two enzymes have been investigated in depth in the work presented in this thesis.

1.1.3.1 Fatty acid amide hydrolase

The term “fatty acid amide hydrolase” was first published in 1996, when it was conclusively shown that the enzymes previously referred to as oleamide hydrolase and anandamide amidohydrolase are in fact the same molecule (Maurelli *et al.*, 1995; Cravatt *et al.*, 1996). It was also shown that FAAH catalyses the hydrolysis of anandamide faster than that of oleamide (Cravatt *et al.*, 1996). FAAH has many substrates, but exhibits specificity for the long-chain amides of ethanolamine (Schmid *et al.*, 1985).

There are two isoforms of FAAH (1 and 2) which share only approximately 20% DNA sequence homology, although the catalytic site is relatively well conserved (Wei *et al.*, 2006). The two isoforms are differentially expressed across species and tissues, with FAAH-1 expressed across many species, including human and rat, and FAAH-2 existing in humans but not rodents (Wei *et al.*, 2006). In human kidney, liver, lung

and prostate both isoforms are expressed. However, only FAAH-1 levels are high in human brain, small intestine and testis, whereas in the heart FAAH-2 is the predominant form expressed (Wei *et al.*, 2006). In subcutaneous adipose tissue, FAAH-1 and -2 are generally expressed to similar levels, with a slightly greater expression of FAAH-1 in lean humans (Bennetzen *et al.*, 2011). Additionally, the two forms have different substrate specificity, with FAAH-1 having greater hydrolytic activity on anandamide than FAAH-2 (Wei *et al.*, 2006). URB597 is a potent, selective and irreversible inhibitor of both enzyme isoforms, although it is more potent at FAAH-2 (Piomelli *et al.*, 2006; Wei *et al.*, 2006).

FAAH is a membrane-bound enzyme (McKinney and Cravatt, 2005) and, interestingly, FAAH-1 is thought to be expressed on the cytosolic side of the cell membrane, whereas FAAH-2 may reside on the extracellular surface (Wei *et al.*, 2006). FAAH is thought to form homodimers, although this has yet to be confirmed physiologically (Bracey *et al.*, 2002). The location and structure of FAAH, attached to the cell membrane and with several channels, may allow the hydrolysis of anandamide from the extracellular space without it ever reaching the cytosol (McKinney and Cravatt, 2005). For reviews on this topic, see (McKinney and Cravatt, 2005; McPartland *et al.*, 2007).

Under experimental conditions, it has been shown that FAAH may catalyse the condensation of arachidonic acid and ethanolamine, but it is unlikely that this occurs physiologically, given the high energy requirement of this reaction (Schmid *et al.*, 1985; Ueda *et al.*, 1995a; Kurahashi *et al.*, 1997).

1.1.3.2 Monoacylglycerol lipase

MGL was first described in lipid metabolism, many years before the ECS was discovered (Vaughan *et al.*, 1964). Its role in the hydrolysis of 1(3)- and 2-monoacylglycerols is essential for the release of free fatty acids from adipocytes, when required, to maintain energy homeostasis. MGL is able to catalyse the hydrolysis of a range of monoacylglycerols with various chain lengths (C8 to C18) (Labar *et al.*, 2010b). The primary role of MGL in many organs is likely to be in the regulation of the ECS rather than in generalised fatty acid release, particularly in the CNS (Dinh *et al.*, 2002a). The MGL isoform found in human adipose tissue, and many other organs, is a 33kDa protein, which shares 83% primary structure homology between rodents and humans (Karlsson *et al.*, 1997; Dinh *et al.*, 2002a; Ho *et al.*, 2002). X-

ray crystallography revealed MGL to exist as a homodimer, as for FAAH (Labar *et al.*, 2010a).

In adipocytes, MGL catalyses the final step in the lipolysis pathway, in which triacylglycerols are catabolised to glycerol and free fatty acids. It is widely thought that MGL does not represent the rate-limiting step of lipolysis, as it is present in excess in adipocytes. Perhaps as a result of this, the regulation of MGL has not been comprehensively investigated, but other lipases involved in lipolysis have been shown to be extensively regulated at the post-transcriptional level (Lafontan and Langin, 2009; Zechner *et al.*, 2009).

Unlike FAAH, MGL is localised in the cytosol. It has been shown that in adipose tissue homogenates, the majority of MGL activity is found in the supernatant fraction, in large lipid-protein aggregates, after centrifugation (Tornqvist and Belfrage, 1976).

1.1.4 Cannabinoid receptors

The two cloned cannabinoid receptors, CB₁ and CB₂, are G protein-coupled receptors (GPCRs) with seven transmembrane domains (Pertwee *et al.*, 2010). Most of the G proteins linked to the cannabinoid receptors are the inhibitory proteins G_i or G_o, and these act predominantly to inhibit adenylyl cyclase (Mackie, 2008). The cannabinoid type 1 (CB₁) receptor was the first to be cloned (Matsuda *et al.*, 1990) and is principally located in the CNS, where it is highly abundant and appears to participate in negative retrograde signalling (Mackie, 2008). CB₁ receptors have also been identified in many peripheral tissues in humans and laboratory animals, including adipose, cardiac and skeletal muscle (Kurz *et al.*, 2008). In contrast, relatively few CB₂ receptors are found in the CNS, and the majority of CB₂ receptors are expressed by cells with immunological functions, such as leucocytes and microglia (Munro *et al.*, 1993; Cabral *et al.*, 2008).

The CB₁ receptor can be activated by both anandamide and 2-AG, and the endocannabinoids have similar affinities for this receptor, although 2-AG is more potent and anandamide is often considered to be a partial agonist. Similarly, both endocannabinoids are agonists of the CB₂ receptor, although again 2-AG is more potent. Both anandamide and 2-AG have greater affinities for the CB₁ receptor than the CB₂. Noladin ether is a selective CB₁ receptor agonist, virodhamine is a partial CB₂ agonist and a CB₁ receptor antagonist, and NADA is a selective CB₁ receptor agonist.

THC is a partial agonist for the cannabinoid receptors but has high affinity for them (Pertwee and Ross, 2002; Pertwee *et al.*, 2010). By contrast, cannabidiol has low affinity for CB_{1/2} receptors, and is thought to be an antagonist of various endogenous and synthetic cannabinoid receptor ligands, leading to similar effects as cannabinoid receptor antagonists (Mechoulam *et al.*, 2007).

Endocannabinoids are known to bind to a variety of other receptors and channels. One such superfamily is the transient receptor potential (TRP) channels. These transmembrane cation channels are most extensively expressed in nociceptive neurons (Tominaga and Caterina, 2004), but are also found in many other tissues such as skeletal muscle, adipose (Cavuto *et al.*, 2007b) and the CNS (Mezey *et al.*, 2000). In general, the TRP channels respond to various noxious stimuli, including heat, physical damage and chemical messengers (Tominaga and Caterina, 2004). With regard to cannabinoid pharmacology, the vanilloid channels (TRPV) are best characterised to date. TRPV1 is primarily a heat receptor expressed in sensory neurons (Latorre *et al.*, 2007), and its activation has been found to cause vasodilation (Zygmunt *et al.*, 1999), but there is also evidence that TRPV1 has an important role in metabolism, as TRPV1 knockout mice are resistant to the obesity-protective effects of capsaicin when fed a high-fat diet (Zhang *et al.*, 2007). TRPV1 is activated by anandamide, OEA, PEA and cannabidiol, but not efficiently by 2-AG or THC (reviews by Pertwee, 2006; De Petrocellis and Di Marzo, 2010). Other TRP channels with known cannabinoid ligand interactions include TRPV2, TRPV4, TRPM8 and TRPA1 (reviewed by De Petrocellis and Di Marzo, 2010).

Additionally, endocannabinoids are ligands for the nuclear peroxisome proliferator-activated receptors (PPARs) (O'Sullivan *et al.*, 2005; Lenman and Fowler, 2007) and there is evidence that these interactions may be as significant in some cells as CB_{1/2} receptor activation (O'Sullivan, 2007; Pagano *et al.*, 2008). Three PPAR isoforms (α , γ and β (δ)) have been identified to date and these form heterodimers with retinoid X receptors (RXRs) before binding to target gene promoters in order to regulate transcription. The PPARs have key roles in regulating cellular differentiation, lipid metabolism and inflammation, and accordingly they are expressed in the majority of human cells and have many known ligands, particularly fatty acids and their metabolites (for PPAR review see Glass and Ogawa, 2006). PPAR α is highly expressed in metabolically active

tissues, such as liver, adipose, skeletal muscle and heart, and is also relatively abundant in monocytes, lymphocytes and macrophages, reflecting its roles in metabolism and immunity. Generally PPAR α activation is metabolically protective and causes reductions in steatosis and inflammatory processes associated with overfeeding in liver and adipose tissue (for review see Stienstra *et al.*, 2007). PPAR α agonists (fibrates) are used clinically in the treatment of dyslipidaemia. PPAR δ has not been well characterised, but it is relatively highly expressed in brain and adipose tissue. Studies using murine models have indicated that it has an important role in metabolic regulation, and possibly in immunosuppression. Interestingly, both PPAR δ knockout mice and those treated with PPAR δ agonists have reduced adipose tissue mass (Stienstra *et al.*, 2007). PPAR γ is most abundantly expressed in adipose tissue compared to other tissues (Auboeuf *et al.*, 1997) and has an essential role in adipogenesis, lipid storage and glucose metabolism. Accordingly, PPAR γ agonists (thiazolidinediones) have been used therapeutically to increase insulin sensitivity in type 2 diabetes. As with the other isoforms, PPAR γ activation is associated with dampening inflammatory processes (Stienstra *et al.*, 2007). There are at least 2 PPAR γ isoforms which are differentially expressed: PPAR γ 1 which is ubiquitous, and PPAR γ 2 which is relatively specific to adipose tissue (Auboeuf *et al.*, 1997). Anandamide, virodhamine, OEA and PEA have all been shown, to varying degrees, to be PPAR α agonists (Fu *et al.*, 2003; Lo Verme *et al.*, 2005; Sun *et al.*, 2006). To date, OEA is the only cannabinoid related agonist reported for PPAR δ (Fu *et al.*, 2003). Cannabinoid related agonists identified to date for PPAR γ include anandamide, 2-AG, THC, cannabidiol and NADA (Bouaboula *et al.*, 2005; O'Sullivan *et al.*, 2005; Rockwell *et al.*, 2006; O'Sullivan *et al.*, 2009a; O'Sullivan *et al.*, 2009b).

Further to these well recognised endocannabinoid targets, several others have been postulated, including calcium and potassium channels (for review see Pertwee *et al.*, 2010), and serotonergic (Kimura *et al.*, 1998; Xiong *et al.*, 2008) and muscarinic receptors (Christopoulos and Wilson, 2001). In some environments anandamide, 2-AG, THC, PEA and OEA are agonists of the orphan receptor GPR55 (Ryberg *et al.*, 2007), but other studies have questioned the response of GPR55 to anandamide and 2-AG (Lauckner *et al.*, 2008; Henstridge *et al.*, 2009). The inclusion of GPR55 in the ECS will remain under debate until its cannabinoid pharmacology has been conclusively characterised. GPR119 is another

orphan receptor which has been considered with regard to the ECS, as anandamide, PEA and OEA were reported to be agonists (Overton *et al.*, 2006; Chu *et al.*, 2010). However, interactions were only found at higher than physiological concentrations, and as no other cannabinoid ligands have been identified it is thought that GPR119 is not involved in the ECS (Pertwee *et al.*, 2010). Another potential endocannabinoid target is the novel endothelial cannabinoid receptor. This receptor was first postulated in rat mesenteric blood vessels (Jarai *et al.*, 1999) and has yet to be fully characterised. Work to date has shown that it may be a $G_{i/o}$ protein coupled receptor which is activated by anandamide and abnormal cannabidiol (Mukhopadhyay *et al.*, 2002; Begg *et al.*, 2003).

As a further complication when considering the signalling of the ECS, recent research indicates that the CB_1 receptor may form heterodimers with receptors such as adenosine A_{2A} (Carriba *et al.*, 2007), dopamine D_2 (Kearn *et al.*, 2005) and orexin 1 (Ellis *et al.*, 2006). Similarly, there is some evidence that cannabinoid receptors and TRPV channels may co-localise and enhance or inhibit each other's signalling (Hermann *et al.*, 2003; reviewed by Di Marzo and Cristino, 2008). Whether all of these interactions occur physiologically remains to be answered, but they may explain some of the apparently contradictory results that are sometimes found within cannabinoid pharmacology.

1.2 Adipose tissue

The primary function of white adipose tissue is to store lipids when food intake exceeds the body's energy requirement and to subsequently release this energy as required. The triacylglycerols stored in mature adipocytes themselves constitute up to 85% of the total mass of adipose tissue, but adipocytes account for less than half of the total number of cells in white adipose tissue; the remaining cellular number comprises cells such as preadipocytes, fibroblasts, endothelial cells and macrophages (Trayhurn *et al.*, 2006). Sites of adipose tissue deposition in humans fall into two categories: visceral and subcutaneous. Visceral adipose tissue is largely restricted to the omentum in healthy people, whereas subcutaneous adipose tissue is found throughout the body directly under the skin. These two major adipose tissue depots are widely thought to be metabolically distinct (for extensive review see Wajchenberg, 2000) and this concept is discussed with reference to various findings throughout the remainder of this chapter.

1.2.1 Lipid metabolism

Lipids are transported in blood plasma in several forms, including free cholesterol, lipoproteins, triacylglycerols and free fatty acids bound to albumin. These molecules are then transported in and out of mature adipocytes as required in the form of free fatty acids and monoacylglycerols. During lipogenesis, lipoprotein lipase is recruited to the endothelium of capillaries and triacylglycerols are catabolised. Fatty acids accumulated in adipocytes from the blood are then re-esterified through the glycerol 3-phosphate pathway to triacylglycerols (see Figure 1.5). These triacylglycerols are stored in a large lipid droplet surrounded by a phospholipid monolayer, which has various proteins embedded in it (for review see Zweytick *et al.*, 2000).

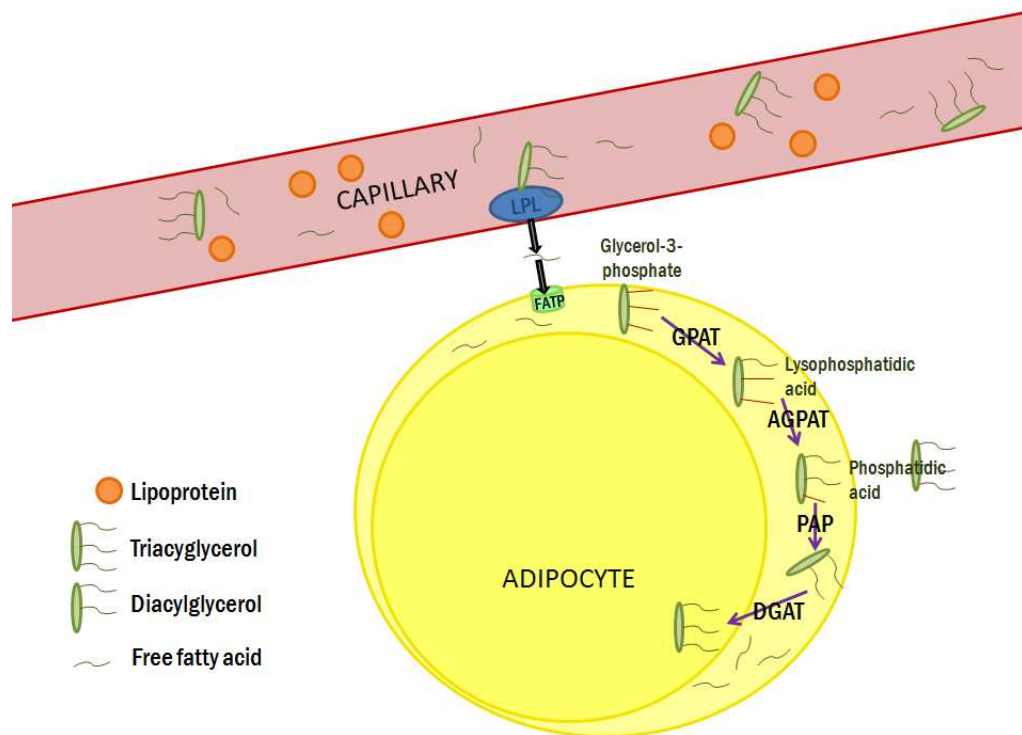


Figure 1.5 Lipid uptake by adipocytes

Simplified schematic of fatty acid uptake from the circulation and some stages of lipid metabolism in the adipocyte. LPL, lipoprotein lipase; FATP, fatty acid transport protein; GPAT, glycerol-3-phosphate acyltransferase; AGPAT, acylglycerophosphate acyltransferase; PAP, phosphatidic acid phosphohydrolase; DGAT, diacylglycerol acyltransferase.

Conversely, during times of fasting or excess energy expenditure, lipolysis is activated in adipocytes and fatty acids are released from storage back into circulation. The major lipolysis pathway has been revised

in relatively recent years by the discovery of ATGL (Jenkins *et al.*, 2004; Villena *et al.*, 2004; Zimmermann *et al.*, 2004). It is now thought that the majority of triglycerides are catabolised to diacylglycerols by ATGL, and are then converted to monoacylglycerols and further free fatty acids under the action of HSL. MGL then catalyses the final stage, to produce glycerol and a free fatty acid (see Figure 1.6).

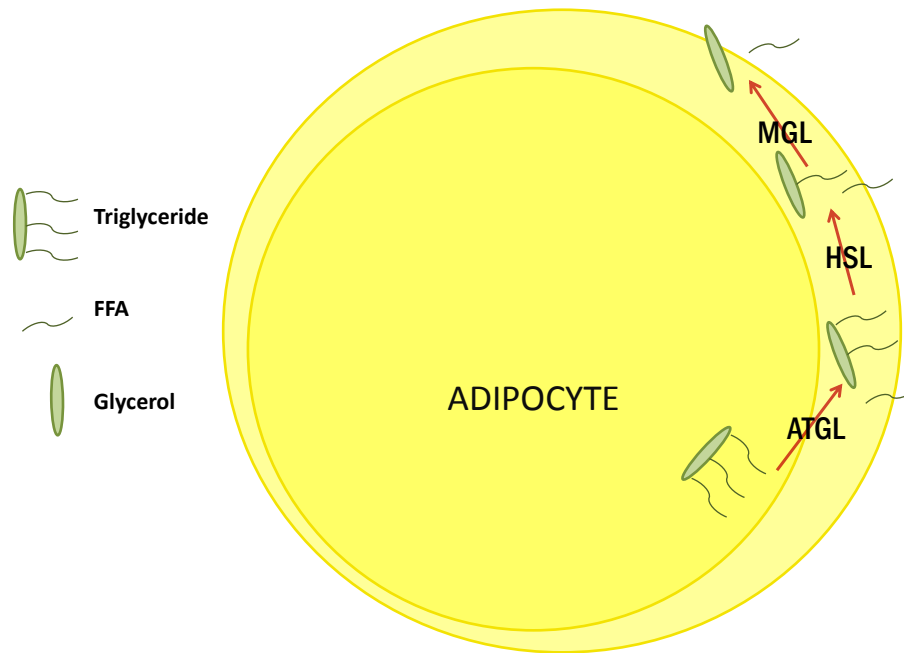


Figure 1.6 Lipolysis

Major lipolysis pathway, involving MGL. FFA, free fatty acid; MGL, monoacylglycerol lipase; HSL, hormone sensitive lipase; ATGL, adipose triglyceride lipase.

1.2.2 The endocrine function of adipose tissue: adipokines

In recent years our understanding of the functions of adipose tissue has developed greatly. Adipose tissue is no longer considered to be merely an inert energy storage facility, but a specialised endocrine organ (Sethi and Vidal-Puig, 2007). Given the total mass of white adipose tissue in healthy humans this is a significant consideration. In healthy men adipose tissue contributes 10-20% to total body mass, whilst in females this figure is 20-30% (Williams and Frühbeck 2009). The signalling proteins, lipids and other factors released by adipose tissue are collectively termed adipokines, although technically adipokines are secreted or modified only by adipocytes (Wang *et al.*, 2008b). The structures and functions of the adipokines are

diverse, and their targets include many organs, such as the skeletal muscle, brain and kidneys (Ronti *et al.*, 2006; Trayhurn *et al.*, 2006). Their effects on these organs are largely metabolic, such as the regulation of fatty acid oxidation and control of appetite, although many adipokines, directly or indirectly, also affect processes such as inflammation, immunity and angiogenesis (Ronti *et al.*, 2006; Trayhurn *et al.*, 2006). In addition, some adipokines behave in a paracrine or autocrine fashion and feedback directly on adipose and its associated tissues (Wang *et al.*, 2008b). Some of the most extensively investigated adipokines involved in energy homeostasis are described in the following paragraphs.

1.2.2.1 Adiponectin

One of the major adipokines, with regard to energy homeostasis and inflammatory processes, is the peptide hormone adiponectin. Adiponectin is a 30 kDa protein which forms trimers, hexamers and larger oligomers (Pajvani *et al.*, 2003). It is produced by mature adipocytes and secreted into the blood in relatively large concentrations compared to many other adipokines (Ronti *et al.*, 2006). Adiponectin secretion and its concentration in blood plasma in humans are inversely correlated with the total volume of adipose tissue in the individual (Arita *et al.*, 1999). In mice fed a high-fat content diet, the rate of adiponectin clearance from blood plasma was found to be reduced, indicating that in obesity the production of adiponectin is likely to be suppressed (Halberg *et al.*, 2009). Females have a higher concentration of circulating adiponectin than males (Arita *et al.*, 1999; Nishizawa *et al.*, 2002) and murine experiments have indicated that this may be due to an increased rate of production, as liver clearance rates are similar (Halberg *et al.*, 2009). Other experiments in mice and cell culture indicate that this difference may be mediated by testosterone (Nishizawa *et al.*, 2002). In contrast with many metabolic mediators, some studies have not found any diurnal variation in plasma adiponectin concentrations in lean or obese humans (Hotta *et al.*, 2000; Yildiz *et al.*, 2004). However, a detailed study using more frequent blood sampling has found that adiponectin concentrations fall overnight (Gavrila *et al.*, 2003).

The importance of adiponectin in maintaining insulin sensitivity is demonstrated by the finding that, in healthy humans, serum adiponectin concentration is negatively correlated with the risk of developing type 2 diabetes, and this predictor was found to be independent of BMI (Spranger *et al.*, 2003). Furthermore, in obese humans, adiponectin secretion from

omental adipocytes is inversely correlated with fasting plasma glucose concentration (Bakker *et al.*, 2006). Adiponectin receptors have been identified in organs targeted by insulin, such as adipose tissue, the liver and skeletal muscle (Wang *et al.*, 2008b). To date, two adiponectin receptors have been identified: AdipoR1, which is ubiquitously expressed, and AdipoR2, which is primarily found in the liver (Williams and Frühbeck, 2009). In mice, AdipoR1 has been shown to activate AMP-activated protein kinase (AMPK), whereas AdipoR2 was found to be linked to PPAR α signalling (Yamauchi *et al.*, 2007). The net effects of activation of these two receptors therefore include increased glucose uptake and fatty acid oxidation in the liver and skeletal muscle, and suppression of hepatic gluconeogenesis, inflammatory processes and ectopic triglyceride storage (Robinson *et al.*, 2011).

1.2.2.2 Leptin

Another key adipokine is leptin, a 16 kDa polypeptide hormone which suppresses appetite and regulates lipid metabolism, primarily via its receptors in the hypothalamus (Hochberg and Hochberg, 2010). In adipocytes, its actions include the activation of fatty acid oxidation and lipolysis, and the downregulation of lipogenesis (Scherer and Buettner, 2011).

Leptin secretion is increased in obesity and its blood plasma concentration correlates with BMI. It has been suggested that the primary role of leptin is actually to stimulate feeding during times of starvation, as plasma leptin concentrations fall during periods of both chronic (Baranowska *et al.*, 2008) and acute (overnight) anorexia (Gavrila *et al.*, 2003). There is mounting evidence that leptin regulates energy homeostasis primarily via its signalling in the brain, particularly in the hypothalamus (Scherer and Buettner, 2011). Systemic disruption of leptin signalling in rodents causes obesity, and several rodent models of obesity (for example ob/ob mice and fa/fa Zucker rats) have mutations in the leptin receptor (Zhang *et al.*, 1994; Ogawa *et al.*, 1995). By contrast, in mice, deletion of peripheral leptin receptors, but not those in the brain, does not appear to affect energy homeostasis or adipose tissue deposition (Guo *et al.*, 2007).

In humans, congenital leptin deficiency is rare, but causes obesity and severe dyslipidaemia. This finding that both insufficient and excess concentrations of leptin in the circulation are associated with similar

pathologies has led to the suggestion of leptin resistance. This concept has yet to be conclusively proven, but it is a theory which explains many of the relationships between obesity and leptin (Heymsfield *et al.*, 1999; Ronti *et al.*, 2006; Oswal and Yeo, 2010). In the pathological state of chronic overfeeding, plasma concentrations of leptin rise and resistance occurs. This reduces anorexigenic signalling and inhibition of lipolysis, leading to a positive feedback loop resulting in increased adipose tissue mass and further resistance (Oswal and Yeo, 2010). In keeping with this hypothesis, a recent review found that even moderate weight-loss in obese humans, via calorie restriction, markedly reduced serum leptin concentration before the secretion of other adipokines was detectably affected (Klempel and Varady, 2011). This may be partly explained by the finding that in mice hypertriglyceridaemia, which is common in obesity, inhibited leptin transport in the brain, further reducing leptin signalling (Banks *et al.*, 2004).

The importance of leptin sensitivity in maintaining normal physiology has also been demonstrated in cell culture, as leptin improves insulin sensitivity and glucose handling in leptin sensitive cells (Ronti *et al.*, 2006). This relationship between leptin and glycaemic regulation is further shown by the finding that leptin secretion by omental adipocytes isolated from obese humans correlates with fasting plasma glucose concentration (Bakker *et al.*, 2006), although it should be noted that adipocyte size is thought to be the most important regulator of leptin secretion .

1.2.2.3 Resistin

Resistin is a 12.5 kDa peptide which was first described in 2001 (Steppan *et al.*, 2001). This protein is a pro-inflammatory mediator which has been shown to increase inflammatory cytokine production and release from several cells, including adipocytes, leucocytes and endothelial cells (for review see Stofkova, 2010). Initially, resistin was thought to be secreted by adipocytes, but it has been shown that the presence of resistin in adipose tissue is largely due to preadipocytes (Janke *et al.*, 2002), leucocytes (Kaser *et al.*, 2003), and other non-adipose tissue cells (Fain *et al.*, 2003) rather than mature adipocytes.

Cell culture work conducted in murine adipocytes and 3T3-L1 cells has shown that resistin expression was induced during preadipocyte differentiation, but also that it inhibited differentiation (Kim *et al.*, 2001). These findings are not supported by a study using human adipocytes

isolated from subcutaneous and omental adipose tissue, which found that resistin release from adipose tissue was not accounted for by mature adipocytes (Fain *et al.*, 2003). Further work in 3T3-L1 cells, indicating a role for resistin in glycaemic regulation and insulin resistance, has shown resistin mRNA to be upregulated by glucose (Shojima *et al.*, 2002) and decreased by insulin (Haugen *et al.*, 2001; Shojima *et al.*, 2002; Liu *et al.*, 2008). Conversely, the effect of resistin on 3T3-L1 cells was to decrease insulin-stimulated glucose uptake (Steppan *et al.*, 2001).

Some authors have suggested that resistin may mediate insulin resistance. Serum resistin concentration may be elevated in obese insulin resistant humans compared to obese insulin sensitive humans (Baranova *et al.*, 2006), but this finding followed a study showing that serum resistin concentrations are only related to insulin sensitivity in lean humans (Heilbronn *et al.*, 2004). Other studies found no difference in serum resistin concentrations (Kielstein *et al.*, 2003; Chen *et al.*, 2006) or adipose tissue resistin mRNA levels (Janke *et al.*, 2002) between healthy, insulin resistant and diabetic patients. Furthermore, the relationship between resistin and obesity remains unclear. In some studies, blood plasma concentrations of resistin were found to be increased in obese mice (Steppan *et al.*, 2001) and humans (Degawa-Yamauchi *et al.*, 2003; Piestrzeniewicz *et al.*, 2008), with corresponding increases in both resistin mRNA (Savage *et al.*, 2001) and protein (Degawa-Yamauchi *et al.*, 2003) in the adipose tissue of obese humans. Weight loss in obese females was found to correlate with a reduction in fasting serum resistin concentration, although this was accounted for by waist circumference rather than BMI (Valsamakis *et al.*, 2004), possibly indicating that visceral adiposity may be more important in predicting resistin concentrations than generalised obesity. This was also supported by a study showing that omental adipose tissue in culture secreted 2.5-fold more resistin than subcutaneous adipose tissue (Fain *et al.*, 2003). By contrast, some investigations have not found any relationship between human obesity and serum resistin concentrations (Heilbronn *et al.*, 2004; Anderlova *et al.*, 2007).

Much of the work on resistin to date has been performed in animals and in 3T3-L1 cells and is highly contradictory (reviewed by Schwartz and Lazar, 2011). Further studies will need to be conducted in humans and isolated human adipocytes before the role and mechanisms of resistin in human physiology, obesity and diabetes are fully understood.

1.3 Obesity

1.3.1 Obesity

In obesity, adipose tissue becomes hyperplasic and hypertrophic, and, as already described to some extent, this is associated with abnormalities in its function and signalling. The incidence and prevalence of obesity have risen sharply over recent decades. In 2005 an estimated 400 million adults worldwide were obese, with a further 1.6 billion adults classified as overweight (BMI 25-30). By 2015 these figures are expected to rise to 700 million and 2.3 billion respectively (WHO, 2006b). The mortality associated with obesity is significant. According to one report obesity is associated with an average 7 year decreased life expectancy for women, and 6 years in men (Peeters *et al.*, 2003). As survival rates fall with increasing BMI, this has been further assessed as a median survival reduction of 2 to 4 years in the 30-35 BMI range, increasing to 8 to 10 years when BMI is greater than 40 (Whitlock *et al.*, 2009).

Obesity is known to be a causative risk factor in the development of many pathological conditions besides metabolic disease, such as cardiovascular diseases, musculoskeletal disorders and some cancers (Biro and Wien, 2010). The expression of many adipokines and other molecules becomes dysregulated in obesity and these changes have roles in the development of many of the diseases associated with obesity. In particular, high concentrations of free fatty acids in the circulation are thought to affect the liver, skeletal muscle, pancreas and heart resulting in increased glucose production, insulin resistance and β -cell damage (Williams and Frühbeck, 2009).

1.3.2 Type 2 diabetes

Type 2 diabetes is a common disease that was estimated to affect approximately 171 million adults globally in 2000, with this figure expected to at least double by the year 2030 (Wild *et al.*, 2004). Type 2 diabetes is characterised by insulin resistance, with pancreatic β -cell failure and elevated plasma glucose concentration (WHO, 2006a), although there are various different local criteria and cut-off points for the diagnosis of diabetes. Obesity is the most significant risk factor in the development of type 2 diabetes. More specifically, excess visceral fat confers a greater risk than the same volume of subcutaneous or lower body fat in the development of type 2 diabetes and other metabolic disorders (Bray *et al.*, 2008; Taksali *et al.*, 2008). In addition, it is visceral adipose mass rather

than total adipose mass that determines the rate of glucose infusion required during a euglycaemic-hyperinsulinaemic clamp (Blüher *et al.*, 2006).

The term metabolic syndrome, although debatable and not internationally defined, is often used in research to describe patients who are at an increased risk of developing type 2 diabetes and cardiovascular diseases (Després and Lemieux, 2006). Different combinations of simple tests and measurements are used to define metabolic syndrome. These include fasting glucose, glucose tolerance, fasting plasma lipids and cholesterol, body mass index (BMI), waist circumference, waist-to-hip ratio and blood pressure (Grundy *et al.*, 2004; Després and Lemieux, 2006; Alberti *et al.*, 2009). It has been argued that waist circumference should be used in preference to BMI, as this better estimates excess visceral adiposity and therefore cardiovascular and metabolic risk (Després and Lemieux, 2006). It is necessary to include metabolic function markers in addition to adiposity measurements in defining metabolic syndrome, as there is a well recognised, but not well characterised, subset of the obese population who do not develop overt metabolic or cardiovascular diseases (Pataky *et al.*, 2010).

Although the correlation between obesity and certain metabolic disorders has been recognised for some time, many of the cellular mechanisms that are responsible for this relationship are still a matter of research.

1.3.3 Dyslipidaemia in obesity

One of the common co-morbidities associated with obesity, and particularly insulin resistance, is dyslipidaemia. Very simply, dyslipidaemia results from alterations in the synthesis and degradation of lipoproteins. In obesity, very low density lipoprotein cholesterol (VLDL-cholesterol) production and high density lipoprotein cholesterol (HDL-cholesterol) degradation both tend to be increased. Additionally, the concentration of triglycerides in the blood is often increased. Dyslipidaemia is thought to arise as a consequence of insulin resistance and increased fat accumulation in the liver and visceral adipose tissue (for recent review see Watts and Chan, 2010).

1.4 The ECS in adipocytes and adipose tissue

It is well established that the ECS is present and functional in human adipose tissue, although to date its precise role and regulation have not been determined.

1.4.1 Endocannabinoids

The basal concentrations of anandamide and 2-AG in human subcutaneous adipocytes in cell culture are approximately equal (Gonthier *et al.*, 2007) and isolated adipocytes from human visceral adipose also contain both endocannabinoids (Matias *et al.*, 2006). It has been shown in a murine preadipocyte cell line that induction of adipogenesis led to a significant increase in intracellular concentrations of anandamide and 2-AG, and that 2-AG concentrations remained high in mature adipocytes compared to preadipocyte levels (Matias *et al.*, 2006; D'eon *et al.*, 2008). In addition, anandamide has been shown to induce differentiation of 3T3-L1 preadipocytes, possibly by direct activation of PPAR γ (Bouaboula *et al.*, 2005). Human subcutaneous adipocytes also produce OEA and PEA. The concentration of OEA is similar to those of anandamide and 2-AG, whereas PEA is present in considerably larger amounts (Gonthier *et al.*, 2007), and this may be of importance when considering metabolic signalling.

1.4.2 Receptors

Functional CB₁ and CB₂ receptors are expressed by preadipocytes and, at higher concentrations, by mature adipocytes from visceral and subcutaneous adipose depots in humans (Roche *et al.*, 2006). Further to this, the CB₁ and CB₂ receptor agonist CP55,940 has been used in studies with 3T3-L1 cells to show that the binding efficiency of the receptors almost doubles 9 days after differentiation is initiated (Gasperi *et al.*, 2007). The relative levels of cannabinoid receptor expression in human adipocytes are a matter of dispute. One study showed CB₂ receptor mRNA levels in mature adipocytes to be approximately 4-fold higher than those of the CB₁ receptor (Roche *et al.*, 2006), whereas another reported CB₂ receptor mRNA levels to fall to almost nothing in human adipocytes after differentiation (Pagano *et al.*, 2007). In human adipocytes, CB₁ receptor mRNA and protein have been reported to be increased in mature adipocytes compared to preadipocytes (Engeli *et al.*, 2005). In 3T3-L1 cells, CB₁ receptor protein expression increases with differentiation,

whereas CB₂ receptor level decreases (Gasperi *et al.*, 2007; Karaliota *et al.*, 2009).

The effects of cannabinoids on adipocytes are summarised in Table 1.1. CB₁ receptor activation with WIN-55,212 in human adipocytes increases glucose uptake via the glucose transporter GLUT-4, even in the absence of insulin. Although WIN-55,212 is a CB₁/CB₂ receptor agonist, the use of the selective CB₁ receptor antagonist rimonabant showed these effects to be CB₁ mediated (Pagano *et al.*, 2007). Similarly, in 3T3-L1 adipocytes, anandamide stimulation, albeit only at a high concentration of 10µM, for 24 hours caused an increase in insulin-stimulated glucose uptake but did not alter the basal glucose uptake rate (Gasperi *et al.*, 2007). Interestingly, in this study, rimonabant only partially blocked the effect of anandamide. Furthermore, anandamide did not affect glucose uptake at the 4 hour time-point (Gasperi *et al.*, 2007), possibly implicating the involvement of receptors with longer response times, such as the PPARs.

In primary culture of murine adipocytes, WIN-55,212 was found to increase lipoprotein lipase activity, and rimonabant blocked this effect, showing it to be CB₁ receptor mediated (Cota *et al.*, 2003). *In vivo* this would lead to an increase in fatty acids available for lipogenesis. Furthermore, in 3T3-L1 cells stimulation of cannabinoid receptors using HU-210, a THC analogue, in differentiating adipocytes increased the rate at which lipid droplets formed. The use of a CB₁ receptor antagonist showed this effect to be CB₁ receptor mediated (Matias *et al.*, 2006). In another study using 3T3-L1 cells, THC stimulation was also found to increase lipogenesis and inhibit lipolysis, and these effects were at least partially blocked using cannabinoid receptor antagonists (Teixeira *et al.*, 2010). There is also evidence that other cannabinoid targets may have roles in the regulation of lipid metabolism, as TRPV1 activation (using capsaicin) was found to inhibit lipogenesis in cultured 3T3-L1 cells (Zhang *et al.*, 2007). Anandamide has been shown to dose-dependently increase the rate of differentiation of 3T3-L1 cells, as measured by triglyceride accumulation and PPAR γ expression and activity, although these effects may be partially mediated by anandamide metabolites (Bouaboula *et al.*, 2005; Karaliota *et al.*, 2009). Interestingly, in one of these studies, the use of rimonabant showed anandamide mediated lipid droplet accumulation to be independent of the CB₁ receptor (Bouaboula *et al.*, 2005). This indicates that PPAR γ activation may be responsible for the observed increases in

differentiation, as PPAR γ stimulation is known to mediate lipid accumulation (Stienstra *et al.*, 2007).

Effect	References
↑ glucose uptake (basal and insulin stimulated)	Gasperi <i>et al.</i> , 2007; Pagano <i>et al.</i> , 2007
↑ Lipoprotein lipase activity	Cota <i>et al.</i> , 2003
↑ lipid droplet formation / lipogenesis	Matias <i>et al.</i> , 2006; Teixeira <i>et al.</i> , 2010
↑ differentiation	Bouaboula <i>et al.</i> , 2005; Karaliota <i>et al.</i> , 2009
↓ lipolysis	Teixeira <i>et al.</i> , 2010

Table 1.1 Cannabinoids and adipocytes

Effects of endocannabinoids, phytocannabinoids and synthetic cannabinoids in adipocytes.

Taken together, these findings indicate that, along with PPAR γ , CB $_1$ receptor activation in adipocytes *in vivo* might increase the rate of preadipocyte differentiation and triglyceride synthesis (Vettor and Pagano, 2009), providing evidence for a role of the ECS in energy storage and homeostasis.

1.4.3 Enzymes

The enzymes of the ECS are known to be present in adipocytes. The principal enzymes responsible for anandamide and 2-AG synthesis, NAPE-PLD and DAGL respectively, are also both present and active in human adipocytes (Spoto *et al.*, 2006) and 3T3-L1 cells (Gasperi *et al.*, 2007). DAGL has a specific activity approximately 8 times greater than that of NAPE-PLD (Spoto *et al.*, 2006), probably reflecting its role in adipocyte lipid metabolism rather than in ECS signalling.

The expression of FAAH is higher in mature human adipocytes than preadipocytes (Engeli *et al.*, 2005; Spoto *et al.*, 2006). This has been further investigated in 3T3-L1 cells, in which FAAH protein and its activity are increased in differentiated cells compared to fibroblasts (Gasperi *et al.*, 2007). Furthermore, stimulating differentiating 3T3-L1 cells with anandamide causes an increase in FAAH mRNA and protein (Karaliota *et al.*, 2009), although whether this is due to the differentiation effects of anandamide or is a mechanism to decrease the intracellular anandamide concentration is unclear.

MGL is also present in human adipocytes and its activity is considerably higher than that of FAAH (Spoto *et al.*, 2006), which is as would be expected given the role of MGL in lipid metabolism. The mechanisms of MGL regulation in adipocytes remain unknown (Labar *et al.*, 2010b) and the activity of MGL in rat adipocytes was found to be stable, regardless of whether the rats were fed or fasted (Tornqvist *et al.*, 1978).

1.5 The ECS in the CNS and adipose tissue in obesity and diabetes

1.5.1 CNS

The ECS was first described in neurons, as a retrograde signalling system, and indeed, the CB₁ receptor is one of the most abundant receptors in the human brain. It has been recognised for many years that CB₁ receptor agonists, such as THC, stimulate appetite and feeding in humans and this is thought to be largely mediated by the hypothalamus and mesolimbic pathways (for review see Bermudez-Silva *et al.*, 2010).

In the hypothalamus, activation of the ECS suppresses satiety signalling pathways and increases orexigenic signalling (Bermudez-Silva *et al.*, 2010), and there is evidence that endocannabinoid concentrations in the hypothalamus of rats fluctuate with metabolic status. 2-AG in particular has been found to be elevated in fasting rats and suppressed post-prandially (Kirkham *et al.*, 2002). Additionally, anandamide administration directly into the hypothalamus of non-fasted rats increased food intake and this was demonstrated to be CB₁ receptor mediated (Jamshidi and Taylor, 2001). Leptin is known to be crucial in hypothalamic metabolic signalling and its relationship with ECS signalling has been studied in some depth. It has been suggested that endocannabinoids are under the negative regulation of leptin in the hypothalamus, as obese rodents and those with leptin mutations have increased anandamide and 2-AG concentrations which can be reduced with leptin administration (Di Marzo *et al.*, 2001; Di Marzo, 2008).

The ECS is also involved in the reward and behavioural aspects of feeding, via its signalling in the mesolimbic pathways. It has been recognised for many years that cannabinoids increase feeding motivation in humans (for review see Kirkham, 2003) and more recently CB₁ receptor antagonism in rats, in the mesolimbic reward areas of the brain, has been shown to suppress palatable food mediated dopamine release (Melis *et al.*, 2007). Similarly, FAAH inhibition in the brain of rats causes a CB₁ receptor

dependent increase in the intake of palatable foods (Dipatrizio and Simansky, 2008).

1.5.2 Rimonabant and the CB₁ receptor in metabolism and obesity

The study of the effects of the potent and relatively specific CB₁ receptor antagonist, SR141716A (rimonabant), has greatly contributed to a better understanding of the ECS in metabolic regulation. Rimonabant has a higher affinity for the CB₁ receptor than CB₂, and is a higher affinity antagonist at the CB₁ receptor than at any other targets described to date. It should be noted however that rimonabant has been reported to bind to a number of other receptors and channels, including opioid, dopamine, TRPs and PPARs (Pertwee *et al.*, 2010). Additionally, rimonabant is sometimes referred to as an inverse agonist, possibly due to inhibition of basal G-protein activity, and there is evidence that these effects may be non-CB₁ receptor mediated (Breivogel *et al.*, 2001; Savinainen *et al.*, 2003; Cinar and Szucs, 2009). In humans, the effects of rimonabant include appetite suppression and increased energy expenditure, and the drug was therefore licensed in Europe to aid weight loss in obese patients (Van Gaal *et al.*, 2005). Rimonabant has now been withdrawn due to an increased risk of suicide, depressed mood disorders and anxiety, despite the exclusion of patients with a history of depression from the clinical trials (Christensen *et al.*, 2007), but some of the data collected from the clinical trials and animal studies are discussed below.

In the published trials of rimonabant its effects on body mass were modest, with mean weight losses of 2.5-8.6 kg reported in patients with a BMI of at least 27 kg.m⁻² (Van Gaal *et al.*, 2005; Pi-Sunyer *et al.*, 2006; Scheen *et al.*, 2006; Rosenstock *et al.*, 2008; Van Gaal *et al.*, 2008b; Despres *et al.*, 2009; Hollander *et al.*, 2010). These weight losses should be considered against the fact that many of the studies involved calorie restriction and/or exercise regimes, and patients on placebo lost 1.4 to 3.6 kg. Specifically, in one trial only 27.4% of patients lost at least 10% of their body weight after 12 months on a 20mg daily dose of rimonabant (Van Gaal *et al.*, 2005). A two year trial showed that weight loss reached its maximal level at one year, and this was maintained for a second year if rimonabant therapy was continued (Van Gaal *et al.*, 2008b). A follow-up study has shown that the majority of patients return to their pre-treatment BMI within one year of rimonabant cessation (Pi-Sunyer *et al.*, 2006). Food behaviour analyses revealed that patients taking rimonabant had

decreased appetite and desire for highly palatable foods, and adhered to a calorie restricted diet more easily (Scheen *et al.*, 2006). Another CB₁ receptor antagonist, taranabant, predictably produced very similar results to rimonabant in phase III trials, but its development was discontinued in 2010 for the same reasons (Aronne *et al.*, 2010; Kipnes *et al.*, 2010; Wadden *et al.*, 2010).

Given that waist circumference is used as a clinical marker of visceral obesity, it has been reported in many of the rimonabant studies. In keeping with the BMI data, rimonabant caused a reduction in mean waist circumference of between 5.2 and 9.1 cm (Despres *et al.*, 2005; Van Gaal *et al.*, 2005; Pi-Sunyer *et al.*, 2006; Scheen *et al.*, 2006; Van Gaal *et al.*, 2008a; Van Gaal *et al.*, 2008b). Interestingly, rimonabant was found to reduce waist circumference more significantly than would be predicted by weight loss alone. Fasting blood concentrations of various metabolic markers such as glucose, HDL-cholesterol and triglycerides were measured throughout the trials and, as with waist circumference, the studies reported larger improvements in these markers than could be accounted for solely by a body mass reduction of 5-10%. For example, the mean circulating triglyceride concentration in one study was reduced by 6.8% in patients treated with rimonabant, compared to a rise in the placebo group, and it was calculated that only 45% of this decrease could be attributed to loss of adipose tissue (Van Gaal *et al.*, 2005). These findings imply the involvement of peripheral organs and tissues in addition to the CNS (Van Gaal *et al.*, 2005). This has also been demonstrated in several animal studies and is described in more detail towards the end of this section, although the precise mechanisms of rimonabant's effects remain under question.

As a result of these findings, a trial was established to assess the glycaemic regulating effects of rimonabant in overweight (BMI ≥ 27 kg.m²) patients with type 2 diabetes not adequately controlled by metformin or sulphonylurea (Scheen *et al.*, 2006). This study found that haemoglobin A1c (HbA1c), a marker of medium-long term glycaemia, was reduced by rimonabant therapy over 1 year, with a value <6.5 in 43% of patients compared to 21% in the placebo group. Similar improvements were reported in fasting glucose concentration and calculated insulin resistance (HOMA-IR), although not in fasting blood insulin concentration. As with other metabolic changes observed in patients given rimonabant, these

improvements could not be entirely accounted for by adipose tissue loss (Scheen *et al.*, 2006).

A further result of the preliminary rimonabant trials was that a large multicentre trial ($n=18,695$) was established to determine the benefit, if any, of long-term rimonabant therapy ($20 \text{ mg}\cdot\text{day}^{-1}$) in the prevention of stroke, myocardial infarction and death due to cardiovascular events. All participants were obese and had either known cardiovascular disease (such as myocardial infarction or stroke) or increased risk of developing cardiovascular disease (including type 2 diabetes and renal artery disease). The trial was stopped prematurely when rimonabant was withdrawn, but data analysed at this point showed no significant difference between rimonabant and placebo (Topol *et al.*, 2010).

Rimonabant has been used in many animal studies to try to determine its precise modes of action. In one such study obese (ob/ob) mice were given rimonabant ($10 \text{ mg}\cdot\text{kg}^{-1}$) daily for 7 days. The treated mice initially reduced their food intake and lost body fat, as has been found in humans, but a novel finding was that their oxygen consumption was significantly increased. Oxygen consumption is a marker of energy expenditure and thermogenesis and this may explain why humans and animals in other studies of CB₁ receptor antagonists maintain their initial weight loss even when hypophagia ceases (Liu *et al.*, 2005). Similarly, in another study, rats fed the same amount as paired rats on rimonabant dropped to the same body weight, but the rats on rimonabant had a significantly lower body fat percentage after 17 days of treatment (Cota *et al.*, 2009). The significance of the CB₁ receptor in feeding can also be seen in CB₁ knockout mice, who consume less food than wild-type controls. Feeding the normal mice the same amounts as consumed by CB₁^{-/-} mice brought about significant weight loss in young mice, but not in adult mice (Cota *et al.*, 2003). These results are further evidence that the metabolic effects of rimonabant are not restricted to appetite suppression and diet-related weight loss.

In cell culture it has been found that rimonabant inhibits proliferation of murine preadipocytes and increases markers of adipocyte maturation without increasing the lipid content of the cells (Gary-Bobo *et al.*, 2006). The team who conducted this research hypothesise that this may help to reduce adipocyte mass and, by returning the adipocytes towards a more normal morphology, restore the homeostatic function of

adipose tissue. In agreement with this, rimonabant has been found to halve the cell size of adipocytes in obese mice (Jbilo *et al.*, 2005).

Several studies have investigated the adiposity and metabolic effects of peripherally restricted CB₁ receptor antagonists such as LH-21 (Pavon *et al.*, 2006; Pavon *et al.*, 2008), AM6545 (Tam *et al.*, 2010) and Compound-1 (Son *et al.*, 2010). In rodents it has been reported that these drugs also cause moderate weight loss and, in some cases, improvements in metabolic profiles, albeit not as efficaciously as rimonabant. However, the findings of the earlier studies have been questioned by other research indicating that LH-21 may be capable of crossing the blood-brain barrier in appreciable quantities and is not CB₁ receptor specific (Chen *et al.*, 2008). In another study designed to measure the effects of peripheral and central CB₁ receptor antagonism, rimonabant was administered either intraperitoneally or intracerebroventricularly in rats. Central CB₁ receptor blockade reduced food intake and body weight, but did not affect metabolic markers, whereas peripheral administration did not affect feeding or body weight but did result in beneficial metabolic changes, particularly in obese animals (Nogueiras *et al.*, 2008). Further pharmacological characterisation of the novel compounds will be required before firm conclusions on the effects of peripheral CB₁ receptor blockade can be drawn.

1.5.3 Cannabinoid receptor expression in obesity

The effects of obesity on CB₁ receptor expression in adipose tissue have been investigated, but the results to date are highly contradictory. In subcutaneous adipose tissue, several papers show CB₁ receptor mRNA to be reduced in obese subjects compared to lean (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Sarzani *et al.*, 2009; Bennetzen *et al.*, 2010). Specifically, one study reported CB₁ receptor mRNA to be reduced by 34% in subcutaneous abdominal adipose samples from obese postmenopausal women compared to lean controls (Engeli *et al.*, 2005). This has also been shown for CB₁ receptor protein levels in subcutaneous adipose tissue, although again using an exclusively female sample (Bennetzen *et al.*, 2010). Conversely, other research has shown CB₁ receptor mRNA to be increased in the subcutaneous adipose tissue of obese patients compared to lean (Pagano *et al.*, 2007). In contrast to both of these findings, Murdolo and colleagues (2007) found no significant differences in CB₁ or CB₂ receptor mRNA in subcutaneous abdominal adipose tissue between lean and obese groups, and CB₁ receptor mRNA in subcutaneous from

obese and lean humans did not correlate with BMI or metabolic status (Lofgren *et al.*, 2007).

In visceral adipose tissue, there are similar discrepancies as found in the subcutaneous depot. Some research has shown CB₁ receptor mRNA to be increased in the visceral adipose tissue of obese patients compared to lean (Pagano *et al.*, 2007; Bennetzen *et al.*, 2010), and mRNA levels to be positively correlated with BMI (Sarzani *et al.*, 2009). Whereas another study showed that CB₁ receptor mRNA in visceral adipose samples from lean and obese humans is not correlated with BMI or metabolic status (Lofgren *et al.*, 2007). Further to this, CB₁ receptor protein levels were not found to differ between lean and obese humans, and this was reported in the same study which reported mRNA levels to be increased in obesity (Bennetzen *et al.*, 2010). This difference between results and conclusions drawn from mRNA and protein assays highlights the importance of measuring the final protein levels or activity of the receptors instead of, or in addition to, mRNA. Alongside these human studies, a recent unpublished study has shown that adipocyte specific CB₁ receptor knockout mice are resistant to diet-induced obesity, which indicates that adipose tissue ECS signalling can affect whole body metabolism (Mancini *et al.*, 2010).

Studies using paired samples of subcutaneous and visceral adipose tissue have highlighted differences in the expression of CB₁ receptor between these adipose tissue depots. In lean subjects, CB₁ receptor expression and protein levels are higher in subcutaneous adipose tissue compared to visceral (Sarzani *et al.*, 2009; Bennetzen *et al.*, 2010). However, in obese patients, CB₁ receptor mRNA was found to be elevated in the visceral depot when compared to paired subcutaneous samples (Pagano *et al.*, 2007; Sarzani *et al.*, 2009) or not different (Bennetzen *et al.*, 2010). Increases in CB₁ expression in visceral adipose tissue have additionally been demonstrated in subsets of viscerally and subcutaneously obese patients (Blüher *et al.*, 2006).

In some studies, the potential relationship between CB₁ receptor expression in adipose tissue and glycaemic dysregulation has been investigated. A CB₁ receptor mutation has been found to be protective against the development of metabolic syndrome in obese, hypertensive, menopausal women. Carriers of the mutation had lower CB₁ expression levels in perirenal adipose tissue, although the sample size was small and the mechanism of the finding is still under question (Bordicchia *et al.*, 2009). Conversely, sustained hyperinsulinaemia was not found to alter

cannabinoid receptor expression in human adipose tissue (Murdolo *et al.*, 2007). In addition to this, CB₁ receptor deletion in adipocytes in mice has been shown to improve glucose handling during high fat feeding (Mancini *et al.*, 2010).

The role of the cannabinoid receptors in lipid metabolism and systemic energy homeostasis has also been investigated. CB₁ receptor mRNA in adipose tissue was shown to be correlated with fasting blood plasma fatty acid concentrations and CB₂ receptor mRNA with adipose tissue adiponectin mRNA (Murdolo *et al.*, 2007). By contrast, another study reported no relationship between CB₁ receptor mRNA levels in human adipose tissue and BMI, metabolic markers such as fasting blood plasma insulin, glucose and lipids, or adipocyte function (Lofgren *et al.*, 2007). In addition to the cannabinoid receptors, TRPV1 mRNA has been found to be downregulated in visceral adipose tissue in obese males (Zhang *et al.*, 2007).

It should be noted that a recent study found that, although not statistically significant, CB₁ receptor mRNA expression in the stroma-vascular fraction of subcutaneous abdominal adipose tissue was double that found in isolated adipocytes from the same adipose tissue (Bennetzen *et al.*, 2010). This indicates that the activity of the ECS is likely to differ between mature adipocytes and adipose tissue, so the results described in adipose tissue above may not apply to adipocytes.

Considered together, these studies indicate that regulation of the of the endocannabinoid receptors in human adipocytes in health and obesity is still very much a matter of debate.

1.5.4 Endocannabinoid levels in obesity

Blood plasma concentrations of anandamide and 2-AG are increased in otherwise healthy obese human volunteers (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Cote *et al.*, 2007). More specifically, circulating 2-AG concentrations have been shown to correlate with waist circumference, and blood plasma anandamide concentration correlates with BMI (Engeli *et al.*, 2005). In another study, in which subjects were subclassified as subcutaneously or viscerally obese, the most significant rise in circulating 2-AG occurred in those with visceral obesity. However in this study, no significant difference in plasma anandamide was detected between the obese and lean subjects (Blüher *et al.*, 2006).

Initial studies have shown that the dysregulation of 2-AG levels may be at least partially reversible. The reduction of visceral adipose tissue volume in viscerally obese men, as measured by body mass, waist circumference and computed tomography, through diet improvement and increased activity, led to a 62% decrease in circulating 2-AG concentrations (Di Marzo *et al.*, 2008). It is likely that weight loss must be substantial for these changes to occur, as other studies have shown that a 5% weight loss in obese humans does not affect blood concentrations of anandamide or 2-AG (Engeli *et al.*, 2005), or mRNA expression of FAAH, MGL or CB₁ receptor in subcutaneous adipose tissue (Engeli *et al.*, 2008).

When considering these results, it should be noted that the blood concentrations of anandamide and 2-AG only reach trace levels, and there is no evidence that endocannabinoids are secreted into the blood as signalling or endocrine factors. It may be that elevated blood plasma endocannabinoid concentrations are a result of overflow from various tissues, and therefore a reflection of systemic ECS tone.

1.5.5 FAAH and MGL in obesity

As seen with CB₁ receptor expression, there is currently no consensus as to whether FAAH is up- or down-regulated in adipose tissue in obesity. In several studies, FAAH mRNA levels have been found to be elevated in the subcutaneous adipose tissue of obese compared to lean subjects (Murdolo *et al.*, 2007; Pagano *et al.*, 2007), whilst others have detected decreased FAAH mRNA levels in obese humans (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Kempf *et al.*, 2007).

The importance of FAAH in maintaining normal body weight has been shown via a missense mutation in the FAAH gene, which occurs in 3.6-10.8% of the population (depending on ethnicity) and is associated with obesity (Sipe *et al.*, 2005). Some variants in the promoter section of the gene have also been linked to an increased risk of obesity (Harismendy *et al.*, 2010), but the effects of these mutations on adipose tissue mass may owe more to hypothalamic and CNS signalling than adipose tissue metabolism. Another large study into polymorphisms in the FAAH gene and obesity revealed two single nucleotide polymorphisms (SNPs) which are associated with early onset extreme obesity in children, but did not find any associations between the FAAH polymorphisms identified and adult obesity (Muller *et al.*, 2007). Further to this, another FAAH missense mutation has been shown to prevent metabolic improvements, as

measured by the homeostasis model assessment (HOMA), normally seen after weight loss in obese humans (de Luis *et al.*, 2010b). The relationship between FAAH in adipose tissue and glycaemic regulation has been further explored in a study in which the euglycaemic-hyperinsulinaemic clamp technique was used to maintain hyperinsulinaemia in obese subjects and matched lean controls. This treatment induced a significant increase of FAAH mRNA in the subcutaneous abdominal adipose tissue of lean subjects, but no change in the obese group, leading the authors to suggest that chronic hyperinsulinaemia could contribute to FAAH upregulation in adipose tissue (Murdolo *et al.*, 2007). However, none of these studies has reported FAAH activity, and using mRNA alone it is not possible to conclusively show that protein levels or activity are altered.

Similar findings to the human FAAH mutation associations with obesity have also been reported in FAAH knockout mice. In these animals, although food intake was not increased, visceral adipose mass (and total body mass) was increased compared to wild-type mice, particularly when on a high-fat diet (Tourino *et al.*, 2010). Additionally, triglyceride accumulation in adipose tissue was higher in the FAAH knockout mice and ectopic fat deposition occurred, accompanied by increased plasma insulin, glucose and leptin concentrations (Tourino *et al.*, 2010). These effects of FAAH are clearly systemic, and it would be informative to study the effects of FAAH dysfunction in adipocytes only, in order to further understand the role of FAAH and endocannabinoid signalling in adipocytes with regard to whole body metabolic homeostasis.

As yet, it is unknown whether FAAH expression is different between visceral and subcutaneous adipose tissue. One study reports that the expression of FAAH in humans does not differ between visceral or subcutaneous adipose tissue, even in obesity (Pagano *et al.*, 2007). However, other research shows FAAH mRNA to be upregulated in visceral compared to subcutaneous depots, and this was observed in humans with both subcutaneous and visceral obesity (Blüher *et al.*, 2006).

MGL expression levels have not been widely investigated in human adipose tissue to date. This may be in part due to early reports that MGL is not hormonally regulated and is not the rate-limiting step in lipolysis, however, in a recent study MGL mRNA was found to be elevated in subcutaneous compared to visceral adipose tissue, regardless of BMI (Pagano *et al.*, 2007). This is an area in which data are lacking, and several further studies into the expression and regulation of FAAH and MGL

in human adipose tissue from different depots will need to be completed before a clearer understanding is achieved.

1.6 The role of the ECS in metabolic homeostasis and obesity in other organs

The ECS is involved in systemic metabolic regulation and components of the ECS, particularly the CB₁ receptor, have been identified in all of the organs involved in energy homeostasis and metabolism (Matias and Di Marzo, 2007). Overall, ECS activation seems to be associated with increases in feeding and energy storage. Interestingly, this has been shown to be important in normal physiology from a very early age, as CB₁ receptor activation is crucial in the early suckling response (Mechoulam *et al.*, 2006). In many organs, the precise function of the ECS has yet to be fully elucidated, but, given the potential contribution of ECS signalling in adipose tissue to overall metabolic homeostasis, a brief overview of some of the findings to date are presented here.

1.6.1 Pancreas

Both CB₁ and CB₂ receptors are present in the human pancreas. Research conducted on islets in primary culture indicate that anandamide and other CB₁ receptor agonists can act via the CB₁ receptor to cause the release of insulin and glucagon, regardless of the glucose concentration (Bermudez-Silva *et al.*, 2008). In contrast, CB₂ receptor stimulation in pancreatic cells appears to have an inhibitory effect (Bermudez-Silva *et al.*, 2008). It has been suggested by the authors of this study that the apparent contradiction of a system whereby the same signalling molecule brings about an increase in two opposing hormones may be explained by a 'saving cycle'. This involves maintaining a hyperglycaemic state under the action of glucagon, in order that insulin can mediate the storage of this glucose. Along with a supply of fatty acids, these are the conditions required for adipose tissue expansion, and this is in keeping with the notion that ECS tone is upregulated in obesity. In support of this hypothesis, elevated levels of anandamide and 2-AG have been measured in the pancreas of obese mice fed a high fat content diet (Matias *et al.*, 2006; Starowicz *et al.*, 2008). In a rat cell model of pancreatic islet β -cells in culture it was found that 2-AG and anandamide concentrations were raised by a two hour incubation in high glucose media and this effect was counteracted by insulin. In addition, cells kept under chronic

hyperglycaemic conditions were not affected by insulin, and in fact insulin caused concentrations of anandamide and 2-AG to increase further (Matias *et al.*, 2006). In keeping with this study, another group using a β -cell line reported elevated insulin secretion under the action of cannabinoid receptor agonists in both low and high glucose medium (Chen *et al.*, 2010).

ECS enzymes have also been measured in the pancreas of lean and diet-induced obese mice. In β -cells, DAGL expression was much higher in the obese mice than lean controls, whereas FAAH mRNA levels were lower. The effect of these changes would be to increase endocannabinoid levels, as has been observed (Starowicz *et al.*, 2008).

1.6.2 Skeletal muscle

All of the ECS components are present in mammalian skeletal muscle, but the CB₁ receptor has received the most attention (Cavuto *et al.*, 2007b; Crespillo *et al.*, 2010). The expression of CB₁ receptors is higher than that of CB₂ receptors in human muscle (Cavuto *et al.*, 2007b) and it has been shown in cell cultures of human muscle that CB₁ receptor protein levels increase during differentiation (Eckardt *et al.*, 2009).

In a human study, CB₁ mRNA levels in skeletal muscle were not found to differ between lean and obese subjects, although the sample size was very small (Cavuto *et al.*, 2007b). This finding is at odds with a rodent study which found high fat feeding to increase CB₁ receptor expression (Pagotto *et al.*, 2006). However, another study using obese Zucker rats found CB₁ receptor protein levels in skeletal muscle to be decreased compared to lean controls (Lindborg *et al.*, 2010). CB₂ receptor mRNA levels have also been reported to be downregulated in obese rats (Crespillo *et al.*, 2010).

CB₁ receptor activation or blockade in muscle has been shown by several teams to affect glucose transport. In rodent studies, CB₁ receptor agonism decreased glucose uptake (Lindborg *et al.*, 2010) and antagonists increased glucose uptake (Liu *et al.*, 2005; Lindborg *et al.*, 2010). However, in cultured human muscle cells, anandamide increased both basal and insulin-stimulated glucose uptake (Eckardt *et al.*, 2009). Interestingly, anandamide has also been shown to activate pathways associated with insulin resistance (Eckardt *et al.*, 2009). Further to this, CB₁ receptor antagonism increased markers of fatty acid oxidation in primary cultures of human muscle (Cavuto *et al.*, 2007a). It may be that

species differences account for the discrepancies between these results, and further work in human muscle will need to be conducted to demonstrate the effects of the CB₁ receptor in normal and pathological physiology.

1.6.3 Liver

The CB₁ receptor is present in human liver, whereas CB₂ receptor expression is not detectable in healthy liver, but is expressed in various pathological states, probably due to inflammatory changes (Mallat *et al.*, 2011). Likewise, CB₁ receptor expression is increased in diseases such as carcinoma and cirrhosis and in mice fed high calorie diets (for review see Tam *et al.*, 2011).

Predictably, given the difficulties in obtaining human samples, most of our knowledge of the ECS in the liver comes from animal studies. In mice, hepatic levels of anandamide were found to be increased in animals fed a high-fat diet, even before a significant difference in weight between the high-fat diet mice and normal-diet mice could be detected. This suggests that, at least in the liver, the endocannabinoid system is upregulated before obesity occurs. In addition to this, the wild type mice fed a high-fat diet had higher levels of hepatic anandamide than CB₁ receptor null mice fed the same diet, indicating that the CB₁ receptor is involved in anandamide upregulation (Osei-Hyiaman *et al.*, 2005).

CB₁ agonists have been shown to increase *de novo* lipogenesis in the liver of rodents, and correspondingly, CB₁ antagonists reduced insulin resistance and the rate of lipogenesis (Tam *et al.*, 2011). This has also been demonstrated using human hepatocytes in a model of fatty liver, in which both CB₁ and CB₂ agonists increased the amount of lipid deposition in hepatocytes (De Gottardi *et al.*, 2010).

1.7 Summary

Taken together, the data published to date shows that the ECS is present and functional in human and other mammalian adipose tissue, and in some cases this has also been specifically demonstrated in mature adipocytes. However, there are contradictions between many of the studies published to date, and questions remain over the precise role and signalling effects of the ECS in adipocytes, and how these are affected by obesity and diabetes. This is the key issue addressed in this thesis.

There is evidence that in adipose tissue, mRNA levels of components of the ECS, such as the CB receptors and enzymes, may be up- or down-regulated in human obesity and type 2 diabetes, but many discrepancies between studies are apparent. In the cases of the catabolic enzymes FAAH and MGL, only mRNA levels in adipose tissue have been reported and it is unknown whether the activities of these enzymes are altered with adiposity or metabolic dysfunction. As the post-transcriptional regulation of these enzymes has yet to be fully characterised, it is important to measure their activities to determine whether these are affected by obesity or diabetes.

In all of the human studies examining the ECS and obesity in adipose tissue reported to date, discrete samples of lean and obese people have been compared. As a consequence, there are very few studies available on the ECS in humans in the overweight but pre-obese state.

In the majority of studies published to date with a focus on comparing the ECS in adipose tissue between lean and obese humans, whole adipose tissue samples have been used when reporting gene expression levels. The work presented in this thesis is novel in that it is based on isolated mature adipocytes from whole adipose tissue biopsies. The power of this approach is that it provides a better idea of the catabolism of the endocannabinoids in adipocytes only, and whether this is dysregulated in obesity or metabolic dysfunction. Interference from other cells in adipose tissue, such as macrophages, is excluded. Obesity has been described as a state of chronic inflammation, and this pathology in adipose tissue could have lead to data showing that the ECS is dysregulated in obesity, but it does not provide information on whether this is the case in the adipocytes themselves. Consequently, there is currently very little information on the ECS in human adipocytes taken from biopsies in health, obesity and metabolic disorders.

The termination of endocannabinoid signalling involves the uptake of the endocannabinoids prior to enzymatic degradation. This has not yet been investigated in human adipocytes, and there are therefore no data available on whether endocannabinoid uptake in cultured cells is affected by insulin or glucose. This is an important consideration in understanding the interplay between ECS and metabolic signalling.

Additionally, the effects of anandamide and 2-AG on adipokine secretion in human adipocytes have not been fully reported. In particular, data are not available on the combined effects of endocannabinoids and

models of metabolic dysfunction on adipokine secretion. These questions are addressed in this thesis, as they will help towards an overall understanding of the ECS in adipocytes and the influence of metabolic factors on this.

1.8 Aims

The ECS is known to have a role in metabolic regulation, but this has yet to be fully characterised, so the overall aim of the study was to investigate the ECS, specifically in mature adipocytes, under normal and pathological conditions. This aim was met by working on the following specific aims using rat models, human samples and human cell culture adipocytes.

For the first study three strains of Zucker rat were used: lean, obese and obese diabetic. The primary endpoint was to determine whether FAAH and MGL activities in rat adipocytes differ between lean and obese or obese diabetic rats, as the activity of these enzymes has not yet been considered in obesity in isolated mature adipocytes. An additional aim using these rats was to compare the subcutaneous, visceral and epididymal adipose tissue depots, with regard to FAAH and MGL activities, as discrepancies in similar comparison studies have been found when reporting FAAH and MGL mRNA levels. The final aim was to determine whether insulin or glucose affect adipocyte FAAH or MGL activity, by comparing the healthy, obese (hyperinsulinaemic) and obese diabetic rats. This work is presented in Chapter 3.

The next set of aims was based on human adipocytes, as FAAH and MGL activities have yet to be investigated in isolated mature adipocytes, and mRNA studies have been inconclusive. The primary aim was to determine whether FAAH and MGL activities in adipocytes vary with BMI. In line with this aim, the secondary aim was to investigate these enzyme activities with regard to waist circumference, as a marker of visceral adiposity. Additionally, given the marked differences in metabolic activity between subcutaneous and visceral adipose tissue, FAAH and MGL activities in adipocytes from these two depots were compared. The final aim using isolated human adipocytes was to determine whether there is a difference in enzyme activity between healthy humans and those with diabetes or markers of metabolic syndrome. The results of these aims are presented in Chapters 4 and 5.

The final set of aims involved human subcutaneous adipocytes in culture, in order to determine directly whether insulin or glucose affect

components of the ECS. The first aim was to investigate whether acute or chronic exposure to insulin, glucose or both increases adipokine secretion in the presence of anandamide or 2-AG. The second aim was to determine whether insulin, glucose or both increase anandamide and 2-AG uptake, or FAAH and MGL activities in mature adipocytes.

2. Materials and Methods

2.1 Zucker rats

2.1.1 Rat models of obesity

The protocol used in the rat study was approved by the University of Nottingham Ethical Review Committee, and the procedures used were approved by the UK Home Office Project and Personal License Authority. The three strains of male Zucker rat used were normal (lean), Fatty (obese) and Diabetic Fatty (ZDF, diabetic). The obese rats were first identified in a colony of Zucker rats which spontaneously developed a *fa* mutation in the leptin receptor (Charles River, UK). This mutation leads to overfeeding, a reduction in thermogenesis (Leonard *et al.*, 2005) and the excessive accumulation of triglycerides in adipocytes (Unger, 1997). The rats therefore become obese and develop insulin resistance, hyperinsulinaemia and hypertriglyceridaemia (Charles River, UK). The diabetic rats were also indentified after a mutation, and subsequent selected inbreeding resulted in a line of diabetic rats (Charles River, UK). These rats are slightly less obese than the obese rats, but they become insulin resistant and hyperglycaemic by the age of 7 weeks (Leonard *et al.*, 2005; Metais *et al.*, 2008). The rats then develop type 2 diabetes as the pancreatic beta cells fail to respond sufficiently to the hyperglycaemic state (Leonard *et al.*, 2005; Marsh *et al.*, 2007; Metais *et al.*, 2008). The lean rats are the same strain but do not have these mutations, and were therefore used as healthy controls. All rats were purchased from Charles River, UK.

2.1.2 Animal housing and food

The Zucker rats were purchased at 6 weeks and killed at 10-12 weeks. The rats were housed in a temperature controlled room with a 12 hour light/dark cycle. Standard rat chow and water were available *ad libitum*.

2.1.3 Adipose tissue collection

Rats were killed by blunt trauma to the head followed by cervical dislocation (Schedule 1). Adipose tissue was immediately dissected from the subcutaneous abdominal, visceral and epididymal adipose depots (see Figure 2.1) and immediately stored at -80°C.

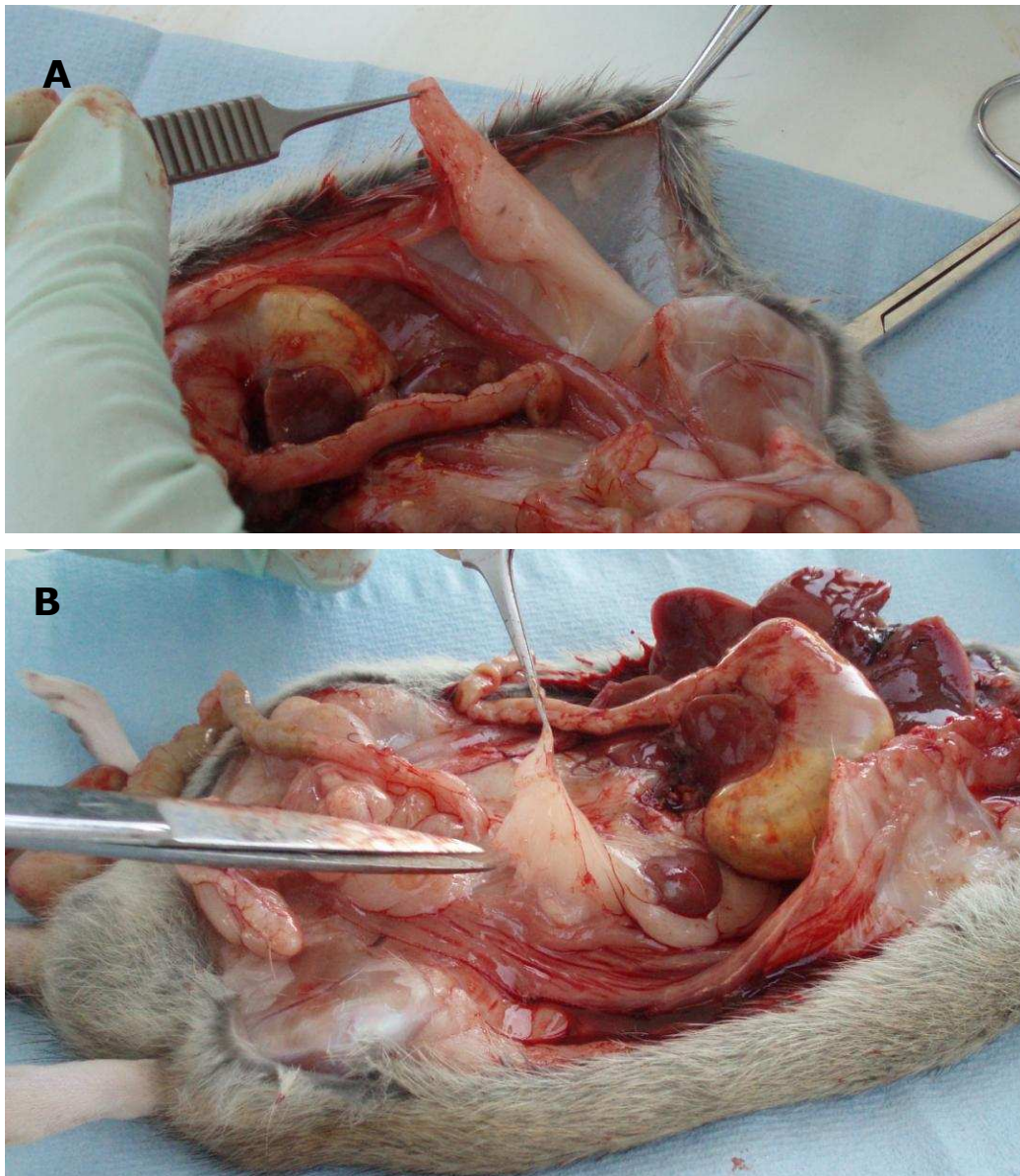


Figure 2.1 Adipose tissue dissection

Dissection of subcutaneous (A) and visceral (B) adipose tissue from Zucker rats.

2.1.4 Blood glucose measurement

In the obese and diabetic rats blood glucose concentration was measured in samples obtained by cardiac puncture immediately after the rats were killed. A drop of blood was added to the testing strip of the Optium Xceed blood glucometer (Abbott Laboratories Ltd., UK).

2.2 Adipocyte isolation and preparation

In order to investigate the ECS in mature adipocytes, rather than whole adipose tissue, the adipose tissue was processed prior to homogenisation.

2.2.1 Purification of mature adipocytes

The method used to isolate mature adipocytes from adipose tissue was adapted from a method published in 1964 (Rodbell, 1964). Each adipose sample was thawed on ice, roughly minced using a scalpel and added to an approximately equal volume of type II collagenase (Sigma-Aldrich, UK, $1\text{mg}\cdot\text{ml}^{-1}$) in PBS. The mix was incubated at 37°C for 45 minutes on a shaking platform to digest the connective tissue, and allow the various cells of adipose tissue to be released. Approximately 20 ml of PBS (room temperature) was then added to the digested tissue and centrifuged (2 minutes, $500 \times g$). The mature adipocytes and any free lipids were found on top of the aqueous phase, whereas all other cellular components remained in the aqueous phase or sedimented to the bottom of the tube. The top layer was carefully removed with a Pasteur pipette and transferred to a clean tube. 10ml of PBS was added, to wash the adipocytes free of any remaining debris, and the cells were centrifuged (2 minutes, $500 \times g$). The floating adipocytes were then collected for homogenisation.

2.2.2 Homogenisation of mature adipocytes

The majority of FAAH is membrane-bound (McKinney and Cravatt, 2005), whereas MGL tends to be cytosolic (Tornqvist and Belfrage, 1976). Therefore, the adipocytes were processed to separate the membrane and cytosolic fractions. The mature adipocytes from each sample were homogenised in approximately 1 ml of Tris-EDTA (TE) buffer using a glass Dounce homogeniser (Uniform, UK). During this process, the homogeniser containing the sample was periodically (1-2 minutes) placed on ice in order to reduce the temperature and therefore the rate of protein degradation. After homogenisation, the mix was centrifuged (10 min, $18,000 \times g$, 5°C) and the supernatant removed. The supernatant was spun again (30 min, $20,000 \times g$, 5°C) and the supernatant from this step, comprising the cytosolic fraction, was then frozen at -80°C for later analysis of FAAH and MGL. The membrane pellet was re-homogenised in 1ml of PBS using a glass homogeniser and centrifuged (30 min, $20,000 \times g$, 5°C). The supernatant from this step was discarded and the pellet resuspended in 1 ml of PBS and stored at -80°C for later analysis.

2.3 Healthy human volunteers

2.3.1 Ethics and screening

Ethical approval was granted by the University of Nottingham Medical School Ethics Committee and informed written consent was obtained from all subjects. Healthy volunteers were recruited from staff and students within the School of Graduate Entry Medicine. The participants were screened using a health questionnaire, and the exclusion criteria included smoking, hypertension and known metabolic disease. All subjects reported a stable weight in the three month period preceding the biopsy. From an initial 34 participants, 6 were excluded due to insufficient biopsy size or fasting blood glucose $>5.6 \text{ mmol.L}^{-1}$, indicating impaired glucose tolerance (American Diabetes Association, 1997).

Volunteers were asked to make two visits. In visit one, anthropometric measurements and blood pressure were taken. In the second visit, no later than one week after visit one, adipose tissue and blood samples were taken.

2.3.2 Anthropometric measurements

Systolic and diastolic blood pressures were measured using an automated digital blood pressure monitor (UA-767, A&D) with the patient rested and in the supine position. Measurements were taken three times and the lowest recorded reading was used for data analysis.

The guidelines followed for taking anthropometric measurements were taken from Norton and Olds (1996). For the skinfold thicknesses, each measurement was taken twice and the mean value was recorded. If the values differed by more than 3 mm then a third reading was obtained and the outlying value was disregarded. Waist circumference was measured at the midpoint between the iliac crest and costal margin. Hip circumference was taken at the widest point around the hips. Neck circumference was measured at the level of the cricothyroid cartilage. Arm circumference was measured at the midpoint between the shoulder and elbow.

Skinfold thickness was measured at 7 anatomical sites using Harpenden callipers, and a tape measure was used to locate the arm sites. The 7 sites were: tricep (posterior, level with circumference), bicep (anterior, level with circumference), subscapular (parallel with inferior angle of scapular), suprailiac (immediately superior to iliac crest), abdominal (2 cm to the side of umbilicus), chest (as high as possible

between anterior axillary line and nipple) and midaxillary (on midaxillary line, level of xiphoid process of sternum) (Norton and Olds, 1996).

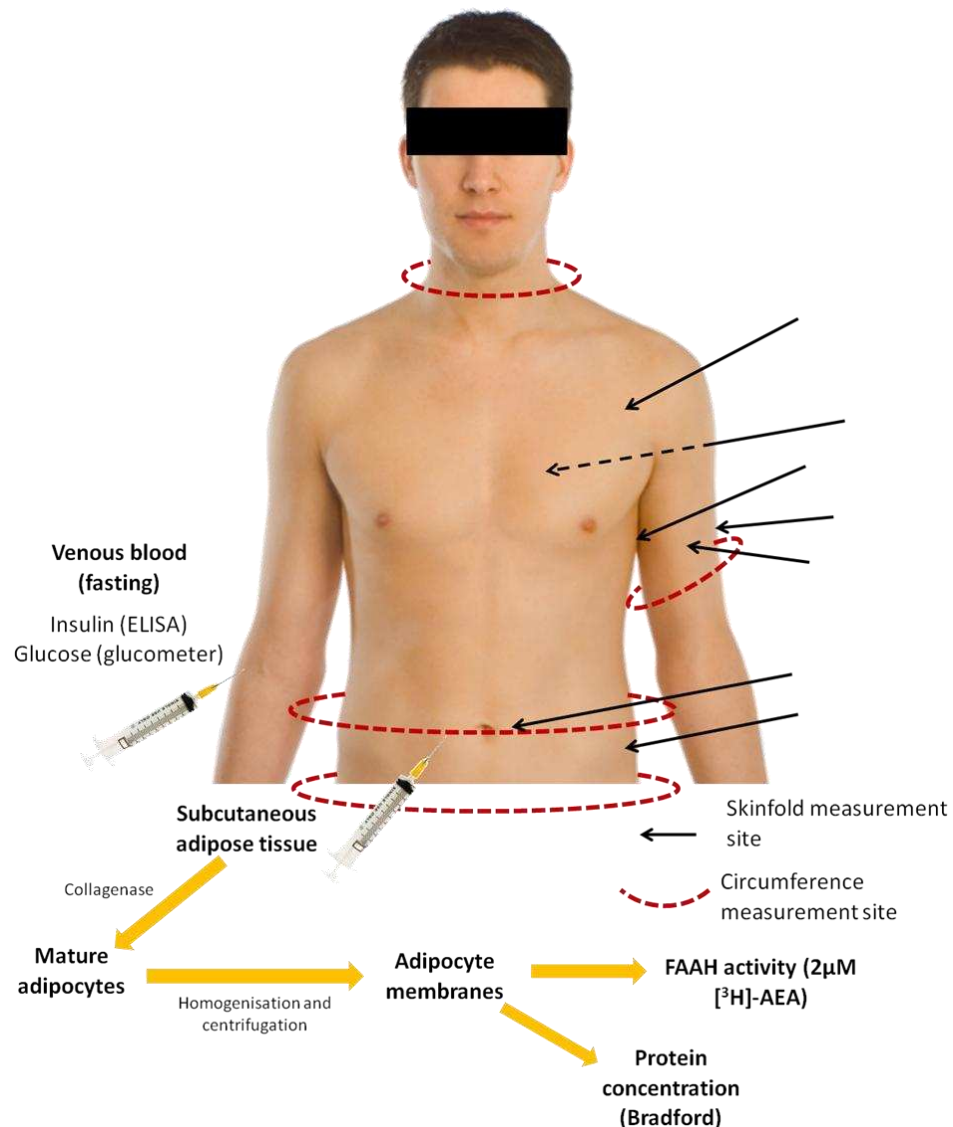


Figure 2.2 Anthropometric measurement sites

Some authors have questioned the reliability and reproducibility of skinfold thickness as an accurate estimate of total body adiposity, but in a study designed to test this, the difference in total body fat percentage calculated from accurate and inaccurate skinfold sites was found to be 1-3% (Durnin *et al.*, 1997).

Various equations have been published over the past 4 decades for the calculation of body fat percentage from skinfold thickness measurements, although these are all broadly similar. The equation used for the work presented here is based on skinfold thickness at the bicep,

tricep, subscapular and suprailiac sites. Table 2.1 shows how body density (D) values were calculated (Durnin and Womersley, 1974) before using the Siri equation ($\% \text{ Body Fat} = [495 / \text{Body Density}] - 450$) to convert these to body fat percentages (Siri, 1961).

Age (years)	Male	Female
<17	$D = 1.1533 - (0.0643 \times L)$	$D = 1.1369 - (0.0598 \times L)$
17-19	$D = 1.1620 - (0.0630 \times L)$	$D = 1.1549 - (0.0678 \times L)$
20-29	$D = 1.1631 - (0.0632 \times L)$	$D = 1.1599 - (0.0717 \times L)$
30-39	$D = 1.1422 - (0.0544 \times L)$	$D = 1.1423 - (0.0632 \times L)$
40 -49	$D = 1.1620 - (0.0700 \times L)$	$D = 1.1333 - (0.0612 \times L)$
> 50	$D = 1.1715 - (0.0779 \times L)$	$D = 1.1339 - (0.0645 \times L)$

Table 2.1 Body density formulae

Equations for the calculation of body density (D) from the log of the total of four skinfold thicknesses (L).

2.3.3 Adipose tissue collection

Volunteers were asked to fast for at least 12 hours prior to their second visit, in which an abdominal subcutaneous adipose sample was taken by trained medical practitioners (Dr Garry Tan or Miss Hye-Chung Kwak). For the adipose tissue biopsy, the area was sterilised using iodine, and local anaesthetic (1% lidocaine) was injected subcutaneously into the biopsy area using a 5 ml syringe fitted with a 21 gauge needle. After 5-10 minutes, a 14 gauge needle fitted to a 50 ml syringe was inserted under the skin and a vacuum was created in the syringe by drawing back the plunger. The adipose tissue biopsies, 1-5 ml in volume, were then aspirated. The biopsy procedure was tolerated well by all volunteers. All volunteers were asked to report back any problems in the hours and days after the biopsy procedure. One volunteer reported extensive bruising (haematoma) which resolved without treatment.

The adipose tissue biopsy was immediately added to an approximately equal volume of type II collagenase, incubated at 37°C and the mature adipocytes isolated as described in Section 2.2.1. Once the mature adipocytes had been isolated and washed, they were stored at -80°C and later homogenised as described in Section 2.2.2.

2.3.4 Blood collection

During the second visit, a fasting venous blood sample (20ml) was taken from each volunteer. The blood samples were transferred to Serum Separator Tubes (SST) with a clotting activator and gel for serum separation (BD Vacutainer System, USA) and allowed to clot (30 minutes). The tubes were then centrifuged ($1000 \times g$, 10 minutes). The serum was immediately removed and stored in aliquots at -80°C . The remainder of each blood sample was discarded.

2.4 Surgical patients

2.4.1 Ethics for surgical study

This study was reviewed and approved by Derbyshire Regional Ethics Committee and Royal Derby Hospital Trust. This cohort included patients undergoing elective laparoscopic bariatric or cholecystectomy surgery at Royal Derby Hospital during the period March 2009 to June 2010. Informed written consent was obtained from the patients in accordance with Good Clinical Practice guidelines by trained staff.

2.4.2 Anthropometric measurements

Anthropometric measurements were taken using the same protocol as the healthy volunteers (see Section 2.3.2) by Miss Hye-Chung Kwak. These measurements were taken within the three months preceding surgery and fasting blood serum profiles were obtained from hospital records within the same period.

2.4.3 Adipose tissue collection

Omental adipose tissue samples (1-5 g) were dissected during surgery using an Endoloop Ligature (Ethicon Endo-Surgery, USA) and scissors, which allowed haemostasis to be achieved using diathermy after the removal of the biopsy rather than during its removal. Towards the end of surgery, the site of the left laparoscopic port is extended to approximately 5 cm to allow the gastric band reservoir to be fitted subcutaneously. During this procedure, 1-5 g of subcutaneous adipose tissue was dissected with scissors and haemostasis achieved using diathermy as necessary. The adipose tissue samples were stored within one hour at -80°C . The samples were thawed, digested and homogenised at a later date as described in Section 2.2.

2.5 Enzyme activity assays

Enzyme activity was measured in this project to give an indication of the rate of anandamide and 2-AG degradation within mature adipocytes at the time-point when the biopsy was taken.

2.5.1 Fatty acid amide hydrolase (FAAH) activity assay

The rat adipocytes fractions (cytosolic and cell particulate) were used in the optimisation and validation stages of the FAAH assay. The results from these assays validated the hypothesis that the majority of FAAH activity would be localised in the cell particulate fraction (McKinney and Cravatt, 2005). This was further demonstrated in a small number of human samples. Thus, in future studies only the cell particulate fraction was used. The use of URB597, a specific FAAH inhibitor, demonstrated that the majority of anandamide hydrolysis measured using this assay was indeed due to FAAH activity.

The assay is designed to detect tritium labelled ethanolamine. FAAH hydrolyses anandamide to arachidonic acid and ethanolamine (see Figure 2.3). Both anandamide and arachidonic acid are adsorbed by activated charcoal, and are therefore sedimented. By contrast, ethanolamine remains in the aqueous phase of the assay and can therefore be easily separated (Wilson *et al.*, 2003; Boldrup *et al.*, 2004).

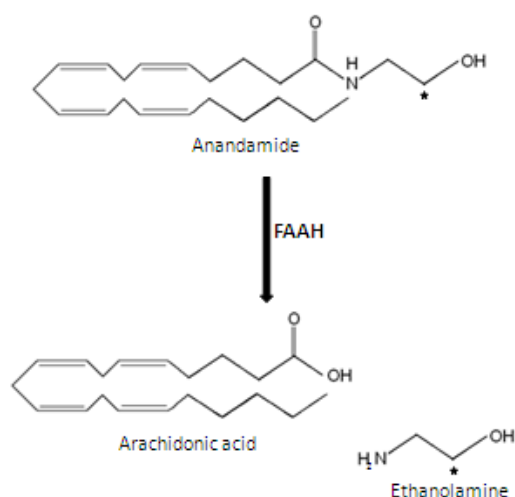


Figure 2.3 FAAH assay

The hydrolysis of tritium labelled anandamide by FAAH (adapted from Wilson *et al.*, 2003).

The method used was adapted from that developed by Boldrup *et al.* (2004). Each homogenate was diluted (1:5) in TE buffer with essentially fatty acid free albumin (1mg.ml⁻¹). 95 µl aliquots of diluted homogenate were added to 1 µl of either URB597 (final concentration 1 µM) or vehicle and incubated at 37°C for 10 minutes in a shaking water bath. 5 µl of [³H]-anandamide (2 µM) was added to each tube and the samples were incubated at 37°C for a further 30 minutes. Activated charcoal (100 µl, 10% w/v in 0.5 M HCl, room temperature) was added to each tube to stop the reaction and the samples were vortexed and left to stand at room temperature for at least 30 minutes. The samples were centrifuged (30 seconds, 1500 x g) and 100 µl of each supernatant was taken for analysis.

The supernatant aliquot from each tube was added to scintillation fluid (3 ml, Ultima Gold XL, Perkin Elmer, USA) and mixed before tritium determination (Tri-Carb 2100 TR Liquid Scintillation Analyser, Packard). Each sample was run in duplicate with vehicle and once with URB597. Tubes without homogenate were run in parallel and used to establish blank values. 5 µl aliquots of the [³H]-anandamide were counted as standards.

The calculation for activity takes into account the amount of tritium in each sample aliquot (sample), the background contamination (blank), the factor for taking a final aliquot, the maximum amount of tritium that could have been in the supernatant (standard), the number of moles of anandamide in each assay (anandamide), the reaction time and the amount of protein in each sample (see Section 2.5.3 for protein assay):

$$\text{Activity} = ([[(\text{Sample} - \text{Blank}) \times 2] / \text{Standard}) \times \text{moles anandamide}] / 30 \text{ minutes}) / \text{mg protein in aliquot}$$

2.5.2 Monoacylglycerol lipase (MGL) activity assay

As with FAAH, the initial validation and optimisation of the MGL assay was performed with the rat samples. The majority of MGL activity was found in the cytosolic fraction of the adipocyte homogenates, so this fraction was used for the MGL assays reported. 2-oleoylglycerol (2-OG) was used in the MGL assay instead of 2-AG, as the two molecules are hydrolysed by MGL at similar rates (Dinh *et al.*, 2002a) and 2-oleoyl-[³H]-glycerol is considerably less expensive than [³H]-2-AG.

Each sample was diluted (1:5) in TE buffer with essentially fatty acid free albumin (1 mg.ml⁻¹) and 95 µl aliquots were incubated for 10 minutes at 37°C with the non-specific MGL inhibitor

methylarachidonylfluorophosphonate (MAFP, 1 μ M) or vehicle. 2-OG (final concentration 100 μ M) was labelled with 2-oleoyl-[3 H]-glycerol and 5 μ l was added to each tube, including blanks containing buffer only. The samples were briefly vortexed and incubated for 15 minutes at 37°C. The reaction was stopped by the addition of 100 μ l activated charcoal (10% w/v in 0.5 M HCl) and the samples left at room temperature for at least 30 minutes. The samples were then briefly centrifuged and 100 μ l of each supernatant was added to 3 ml of scintillation fluid for tritium determination as described in the FAAH activity assay.

The specific activities for MGL were calculated in the same way as for FAAH:

$$\text{Activity} = ([[(\text{Sample} - \text{Blank}) \times 2] / \text{Standard}) \times \text{moles of 2-OG}] / 15 \text{ minutes}) / \text{mg protein in aliquot}$$

2.5.3 Enzyme activity optimisation

Various stages of the enzyme assays were altered to optimise the assays, and some of these are represented in Figure 2.4. 8% charcoal mixture was initially used, and this was increased to 12% to achieve greater separation of substrate and product. The initial MGL assay protocol included a chloroform:methanol step to separate hydrolysed 2-OG, but this gave unacceptably high blanks and so the activated charcoal method was used for the results presented in this thesis. Concentrations of samples ranging from undiluted to 1:50 dilution were tried, with 1:5 dilution reproducibly giving DPM values within the acceptable range. Additional optimisation stages (not shown) included alterations in the length of incubation time, the total volume of the assay and the method used to create the blanks. The aim of these optimisation steps was to reproducibly achieve 5-10% of the standard DPM values in the samples, and to have blank values as low as possible, and in any case no greater than 30% of the sample values.

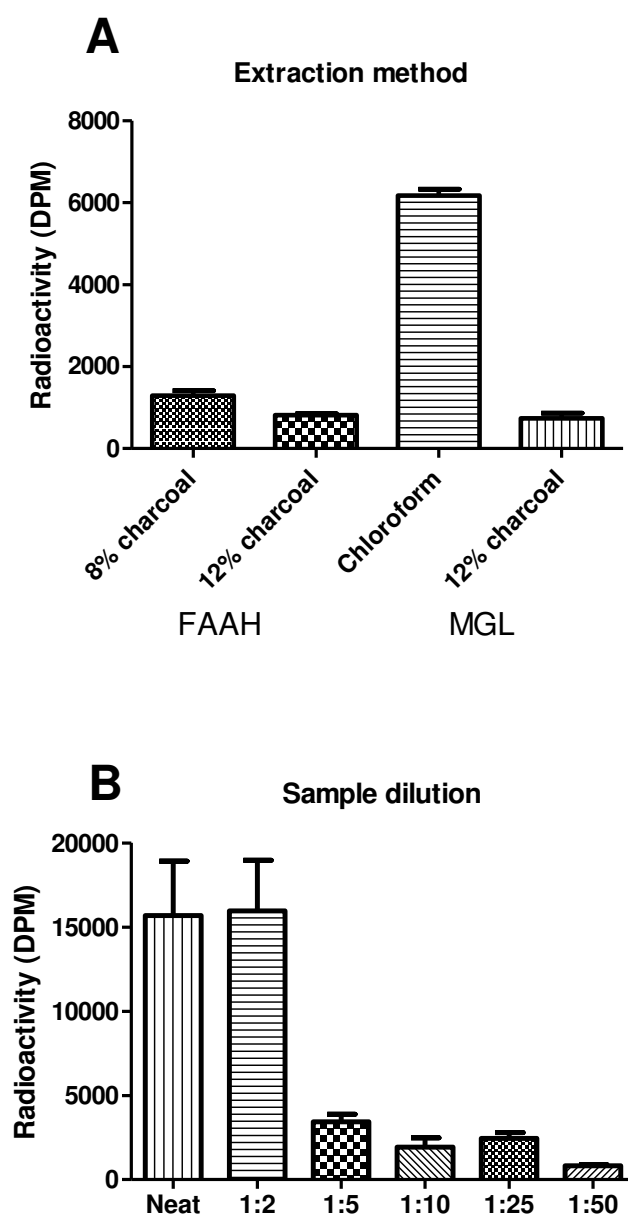


Figure 2.4 Optimisation of FAAH and MGL assays

Some of the optimisation steps in the development of the FAAH and MGL assays. The method of separation of hydrolysed anandamide or 2-OG was optimised using homogenised Zucker rat adipocytes pooled from three adipose tissue depots (A). The dilution of the pooled, homogenised rat adipocyte membranes was optimised. Results are shown for FAAH (B) and were similar for MGL in the cytosolic adipocyte fraction. $n=3-6$. DPM, degradations per minute

2.5.4 Protein assay

The protein concentration of each homogenate fraction was assayed using a commercially available Bradford reagent. 250 μ l of Bradford reagent (Sigma-Aldrich, UK) was added to 5 μ l of each sample or BSA standard in a 96-well plate, which was then read at 595 nm in a spectrophotometer

(Victor 1420 Multilabel Counter, Wallac). All samples and standards were run in duplicate and samples with high protein concentrations were diluted as necessary.

A standard curve for each plate was produced using the mean of the standard duplicates, minus the blank value. Linear regression was used to plot the standard curve and the sample protein concentrations were calculated from this.

2.6 Cannabinoid receptor assays

2.6.1 GTP γ S binding assay

The GTP γ S binding assay has been used by many teams to report on the activity of cannabinoid receptors (Sim *et al.*, 1995; Hosohata *et al.*, 1997; Petitet *et al.*, 1997). The protein concentration of the membrane fraction of pooled rat adipocytes was determined using the Bradford method. This sample was diluted in buffer (50 mM Tris, 100 mM sodium chloride, 10 mM magnesium chloride hexahydrate, 100 μ M GDP, 3.9 mM theophylline and 0.2 mg/ml BSA), to give final protein concentrations of 50, 100 and 200 μ g/ml, as reproducible results within this concentration range had been obtained in several types of tissue by others in the laboratory. The membrane fraction of homogenised rat brain was used as a positive control at a protein concentration of 75 μ g/ml. The membranes were incubated in this buffer at 30°C for 20 minutes, before adding GTP γ S-[³⁵S] (final concentration 20 pM) and GTP γ S (final concentration 10 μ M), WIN-55,212 (CB₁ and CB₂ receptor agonist; final concentrations 10 pM to 100 μ M) or buffer only. The samples were then incubated for a further 90 minutes at 30°C, before the assay mixtures were transferred onto filter paper (Brandell Harvester) and washed four times with cold distilled water. The filter discs were then collected and the radioactivity measured using scintillation fluid (3 ml, Ultima Gold XL, Perkin Elmer, USA) and a scintillation counter (Tri-Carb 2100 TR Liquid Scintillation Analyser, Packard).

2.6.2 CB₁ receptor ELISA

The GTP γ S assay did not give results using the mature adipocyte samples, so a sandwich ELISA was used to try to measure CB₁ receptor protein concentration. Polyclonal antibodies for two epitopes of the CB₁ receptor were purchased (Abcam, UK). Despite several attempts to create a standard curve for this assay, the same level of non-specific, low-level

binding was detected at several concentrations of primary (1, 2 and 5 $\mu\text{g.ml}^{-1}$) and secondary antibody (1:25 and 1:50), and with various concentrations of the CB₁ receptor peptide (0 – 2 $\mu\text{g.ml}^{-1}$). It was therefore concluded that a CB₁ receptor ELISA could not be developed at the time of this study.

2.7 Biochemical assays

2.7.1 Glucose assay

Serum glucose concentrations were determined using the YSI 2300 STAT PLUS glucose and lactate analyser (YSI Life Sciences, USA) within 6 months of sample collection. Quality control (QC) standards were analysed after every 10 samples processed, and, in order to ensure the accuracy of the sample results, samples were not processed unless QC values were within the limits set by the manufacturer.

2.7.2 Insulin assay

The insulin concentrations of the serum samples were measured using a commercial direct sandwich ELISA kit (Mercodia, Sweden) using two monoclonal antibodies. The kit was received as a 96-well plate, pre-coated with the primary antibody. All of the reagents were included in the kit and reconstituted as directed. The standards were received ready-to-use and the human blood serum samples did not require dilution prior to assay.

25 μl of each standard or sample was pipetted into each well, and each standard and sample was assayed in duplicate. 100 μl of the secondary antibody was then immediately added to each well and the plate was incubated on a plate shaker for 60 minutes at room temperature (18-25°C). The plate was then washed 6 times with wash buffer and blotted on a paper towel. 200 μl of tetramethylbenzidine (TMB) was added to each well and the plate was left for 15 minutes at room temperature. 50 μl Stop solution (0.5 M sulphuric acid) was then added to each well and the plate was tapped to ensure thorough mixing. The plate was read on a spectrophotometer (Victor 1420 Multilabel Counter, Wallac) at a wavelength of 450 nm.

The insulin concentrations of the samples were calculated by first subtracting the blank value from all other values. The mean for each set of duplicates was then calculated and the absorption values for the standards were plotted against the insulin concentrations. GraphPad Prism software

was used to perform cubic spline regression analysis on the standard curve and subsequently interpolate the insulin concentrations of the samples.

2.7.3 Adiponectin, leptin and resistin

The concentrations of adiponectin, leptin and resistin were determined using sandwich ELISAs (R&D, UK) according to the manufacturer's instructions. The concentrations of adiponectin, leptin and resistin were measured in the serum samples of the healthy volunteer study. The concentrations of all three cytokines were also measured in cell culture media.

The method for the sandwich ELISA was the same for the measurement of all of these adipokines. All incubations and reagents were at room temperature. The capture antibody was diluted, to the working concentration recommended, in PBS and 100 μ l per well was added to 96-well ELISA plates (Greiner, UK). The plates were sealed and left overnight at room temperature. The plates were then washed (0.05% Tween in PBS) three times, using 300 μ l of wash buffer per well. The plates were blotted against paper towels after the final wash. Non-specific binding was prevented by adding 300 μ l of Reagent Diluent (1% BSA in PBS) to each well for 60 minutes. The plates were then washed three times and the blanks, standards and samples (100 μ l per well) added to the plates in duplicate, and incubated for 2 hours. One set of standards was used on each plate and the standards and samples were diluted in Reagent Diluent as necessary. The plates were then washed three times and the detection antibody (100 μ l per well) was added for 2 hours. After washing again, Streptavidin-HRP was added (100 μ l per well) for 20 minutes, protected from light. The plates were washed and TMB (100 μ l per well) was added for 20 minutes, and protected from light. The conversion of TMB was halted with the addition of sulphuric acid (Stop solution, Sigma-Aldrich, UK; 50 μ l per well). The plates were then read at 450 nm (Victor 1420 Multilabel Counter, Wallac).

The concentrations of the samples were calculated as follows. The mean was taken for all duplicates, and the blank optical density reading was subtracted from all other values. The standard curve was generated with a four parameter logistic curve fit, using Prism GraphPad software. The cytokine concentrations of the samples were then interpolated from the equation of this curve and multiplied by the dilution factor as necessary.

2.8 Cell culture experiments

2.8.1 Adipocyte culture

Human white subcutaneous preadipocytes obtained from females undergoing cosmetic liposuction were purchased from Promocell (Germany), and cultured according to the manufacturer recommendations using aseptic techniques. The Promocell media (Preadipocyte Growth Medium, Preadipocyte Differentiation Medium and Adipocyte Nutrition Medium) were supplemented with penicillin ($100 \text{ units.ml}^{-1}$, Invitrogen, UK) and streptomycin ($100 \text{ } \mu\text{g.ml}^{-1}$, Invitrogen, UK), and the cells were cultured in incubators providing a humidified atmosphere at 37°C with 5% CO_2 .

The cells were received on dry ice and immediately transferred to liquid nitrogen for storage. 12 ml of Preadipocyte Growth Medium in a T75 flask was put in the incubator for 30 minutes. The preadipocytes were then removed from the liquid nitrogen and the vial was gently shaken in a water bath at 37°C . When the medium was 90% thawed, the vial was removed to the laminar flow hood, sprayed with 70% ethanol and the revived preadipocytes were transferred to the pre-incubated T75 flask. The flask was placed in the incubator and the medium replaced after 18 hours. Thereafter, the medium was changed every 2-3 days. The preadipocytes were grown to approximately 70% confluence.

The preadipocytes were split using trypsin and trypsin neutralising solution (TNS, Sciencell, USA) according to the manufacturer recommendations to release the cells from the flask. The preadipocyte monolayer was washed with filtered PBS, and the trypsin (1 ml) added for 3-5 minutes until 80-90% of the preadipocytes were rounded and detached from the flask. TNS (1 ml) was then added and the liquid was transferred to a 15 ml Falcon tube. The flask was washed out with Preadipocyte Growth Medium (5 ml) which was also added to the Falcon tube. The cell suspension was centrifuged (250 g , 2 minutes), the supernatant discarded and the cell pellet resuspended in medium and divided between 4 T75 flasks. The preadipocytes in these were then grown to approximately 70% confluence, before passaging again or seeding plates.

For various experiments, cell culture treated 6-, 24- and 96-well plates were fibronectin (Sigma-Aldrich, UK) coated for 1 hour at 37°C . This was done to increase the adherence of the adipocytes to the plates during the differentiation phase. The plates were then seeded with preadipocytes

at passage no later than 8. At confluence, the medium was changed to Promocell Preadipocyte Differentiation Medium for 72 hours. After this, the cells were cultured in Promocell Adipocyte Nutrition Medium for 12-14 days until differentiation was complete, as assessed by morphological changes. Representative images of the differentiated adipocytes are shown in Figure 2.5.

2.8.1.1 Oil Red O staining of adipocytes

Oil Red O stain stock was prepared by dissolving 0.7 g of Oil Red O in 200 ml of Isopropanol. The solution was stirred overnight, then filtered (0.2 µm filter paper) and stored at 4°C for up to one month. The working solution was prepared by mixing 6 parts stock solution with 4 parts distilled water. The solution was left for 20 minutes before filtering (0.2 µm filter paper).

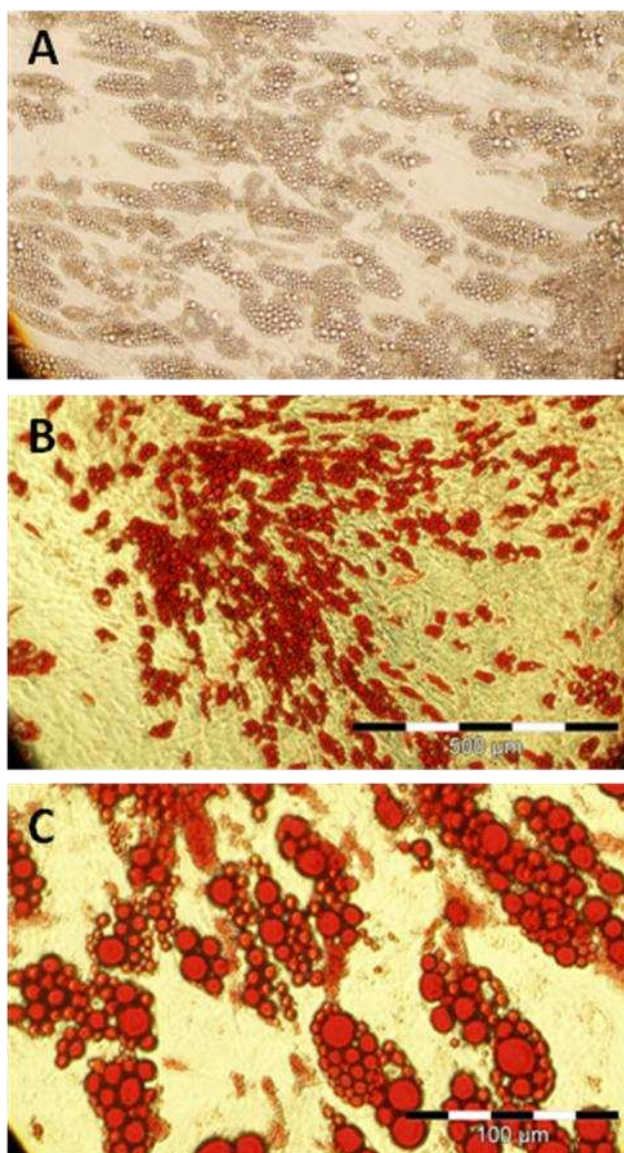


Figure 2.5 Differentiated human subcutaneous adipocytes in culture.

Images are representative of adipocytes unstained (A), stained with Oil Red O to show lipid accumulation (B) and at a higher magnification to show individual adipocytes with multiple lipid droplets. Images were taken 14 days after induction of differentiation.

The cultured adipocytes were washed 3 times with distilled water, then fixed with 4% paraformaldehyde (PFA, 20 minutes). The PFA was removed and the cells were washed once with 60% isopropanol. Sufficient isopropanol to cover the cells was then added and left to evaporate. Oil Red O stain was added (10 minutes) and the plates were then washed 6 times with tap water (see Figure 2.5).

2.8.2 Effects of endocannabinoids on adipokine secretion

The acute and chronic effects of anandamide and 2-AG on adipokine secretion have not been fully characterised, particularly not in models of metabolic dysfunction. These experiments were conducted on human subcutaneous adipocytes, cultured as described in section 2.8.1. The cells were grown in 24-well plates. In the 2 hour endocannabinoid experiments (acute), the Adipocyte Nutrition Medium was changed for medium supplemented with insulin (1 μ M), glucose (15 mM), or both or neither (control). Anandamide, 2-AG or vehicle was added to each well after 22 hours in increasing concentrations (0.01 to 10 μ M). The medium was then harvested at 24 hours and immediately stored at -80°C. For the 24 hour (chronic) experiments, the Adipocyte Nutrition Medium was supplemented with insulin (1 μ M), glucose (15 mM), or both or neither (control). At the same time, anandamide, 2-AG or vehicle was added to each well. After 24 hours the medium was harvested and stored (-80°C). The concentrations of various adipokines in the media were later determined via ELISAs (see section 2.7.3).

2.8.3 Endocannabinoid uptake assay

These experiments were undertaken during a visit to Professor Fowler's laboratory in Sweden and the assay methods used are those of Rakhshan (2000) as modified by Sandberg and Fowler (2005).

Human white preadipocytes (Promocell) were cultured as previously described. 24 well plates were used and half of the wells in the plates were left unseeded, but treated with the same media and buffers, to allow measurement of background endocannabinoid adsorbance. Glucose (15 mM) and/or insulin (1 μ M), when used, were added to the plates after full differentiation of the adipocytes. After treatment, the plates were washed at 37°C with 1% BSA Krebs-Ringer-Hepes (KRH) buffer, and then with KRH buffer. After aspiration of the buffer, 340 μ l of KRH buffer containing 0.1% fatty acid-free BSA was added to each well plus 10 μ l of the FAAH inhibitor URB597 (1 μ M), the MGL inhibitor JZL184 (1 μ M), or vehicle, and the plates were incubated for 10 minutes at 37°C. 50 μ l of [3 H]-anandamide or [3 H]-2-AG in 0.1% fatty acid-free BSA KRH buffer was added to each well and the plates incubated at 37°C for a further 4 minutes. The reaction was stopped by placing the plates on ice and washing the cells three times with cold 1% BSA KRH buffer. After removal of the buffer, sodium hydroxide

(0.2 M, 500 μ l per well) was added and the plates were incubated at 75°C for 15 minutes. The plates were allowed to reach room temperature before aliquots from each well (300 μ l) were transferred to scintillation vials. Scintillation fluid (4 ml per vial) was added and liquid scintillation spectroscopy was used to assess tritium levels.

2.9 Chemicals, solutions and buffers

The following is a table of chemicals, solutions and buffers that were used in the assays that have been described in this chapter:

Product or solution	Company	Components
[³ H]-anandamide	American Radiolabelled Chemicals, USA	
2-AG	Tocris, UK	
2-oleoyl-[³ H]-glycerol	American Radiolabelled Chemicals, USA	
Adipocyte nutrition medium	Promocell, Germany	0.03 ml.ml ⁻¹ fetal calf serum, 8 µg.ml ⁻¹ d-Biotin, 0.5 µg.ml ⁻¹ insulin, 400 ng.ml ⁻¹ dexamethasone
Anandamide	Tocris, UK	
Bovine serum albumin (essentially fatty acid free)	Sigma-Aldrich, UK	
Bovine serum albumin (for ELISA)	Sigma-Aldrich, UK	
Bradford reagent	Sigma-Aldrich, UK	
Collagenase (type II)	Sigma-Aldrich, UK	
DMSO	Sigma-Aldrich, UK	
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, UK	
ELISA wash buffer		0.05% Tween in PBS
ELISA reagent diluent		1% BSA in PBS
Ethanol	Sigma-Aldrich, UK	
Fibronectin	Sigma-Aldrich, UK	
Glucose	Sigma-Aldrich, UK	
Insulin	Sigma-Aldrich, UK	
JZL184	Tocris, UK	
Krebs-Ringer-Hepes buffer (KRH)		120 mM sodium chloride, 4.7 mM, 2.2 mM calcium chloride, 10 mM Hepes, 0.12 mM monopotassium phosphate, 0.12 mM magnesium sulphate, pH 7.4
Phosphate buffer tablets	Sigma-Aldrich, UK	
MAFP	Tocris, UK	
Penicillin-Streptomycin	Invitrogen, UK	

Phosphate buffered saline (PBS)	Sigma-Aldrich, UK	10 mM phosphate, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4
Preadipocyte differentiation medium	Promocell, Germany	8 $\mu\text{g}.\text{ml}^{-1}$ d-Biotin, 0.5 $\mu\text{g}.\text{ml}^{-1}$ insulin, 400 $\text{ng}.\text{ml}^{-1}$ dexamethasone, 44 $\mu\text{g}.\text{ml}^{-1}$ IBMX, 9 $\text{ng}.\text{ml}^{-1}$ l-thyroxine, 3 $\mu\text{g}.\text{ml}^{-1}$ ciglitazone
Preadipocyte growth medium	Promocell, Germany	0.05 $\text{ml}.\text{ml}^{-1}$ fetal calf serum, 4 $\mu\text{l}.\text{ml}^{-1}$ endothelial cell growth supplement, 10 $\text{ng}.\text{ml}^{-1}$ epidermal growth factor, 1 $\mu\text{g}.\text{ml}^{-1}$ hydrocortisone, 90 $\mu\text{g}.\text{ml}^{-1}$ heparin
TE buffer		50mM Tris, 1mM EDTA, pH 7.4
Tris(hydroxymethyl)aminomethane (TRIS)	Sigma-Aldrich, UK	
Trypsin EDTA	Sciencell, USA	0.025% trypsin, 0.5 mM EDTA, 1 mM sodium pyruvate and 10 mM HEPES, pH 7.4
Trypsin neutralising solution	Sciencell, USA	10% fetal calf serum, 0.5 mM EDTA, 1 mM sodium pyruvate and 10 mM HEPES, pH 7.4
Tween20	Sigma-Aldrich, UK	
URB597	Sigma-Aldrich, UK	

2.10 Statistical analysis

All statistical analysis was performed using GraphPad Prism software (GraphPad Software, USA).

2.10.1 Enzyme activity assays

In all analysis where FAAH and MGL activities were compared between three groups, one-way analysis of variance (ANOVA) and Bonferroni's *post hoc* test were used. This applies to the three rat strains (lean, obese and obese-diabetic), the three adipose tissue depots taken from the rats (subcutaneous, abdominal and epididymal) and the three groups of surgical patients (healthy, metabolic syndrome and diabetes). A *P* value <0.05 was deemed to be statistically significant.

In the surgical patient study, enzyme activities were compared between subcutaneous and visceral adipose tissue depots. For this analysis, a paired *t* test was used with *P*<0.05 as statistically significant.

Various correlation studies were performed in the Zucker rat and two human studies, between enzyme activities and anthropometric or blood serum values. Normality was tested for in all data sets before further analysis. The Pearson correlation coefficient (r) was calculated and $P < 0.05$ was taken as statistically significant.

MGL activity was also measured in cells cultured with insulin and/or glucose. These data were analysed using one-way ANOVA and Dunnett's multiple comparison test, with all treatments compared against the basal activity rate and $P < 0.05$ considered to be statistically significant.

2.10.2 Adipocyte culture adipokine secretion

The concentrations of various adipokines in harvested culture medium were compared using one-way ANOVA and Dunnett's multiple comparison test. Various concentrations of anandamide and 2-AG were used in these experiments, and the adipokine secretion for each of these was compared against the secretion measured in vehicle treated cells. $P < 0.05$ was deemed to be statistically significant.

2.10.3 Endocannabinoid uptake

The effects of insulin and glucose on anandamide and 2-AG uptake in cultured adipocytes were compared against basal uptake using one-way ANOVA and Dunnett's multiple comparison test. $P < 0.05$ was deemed to be statistically significant.

3. A study of the ECS in mature adipocytes from lean, obese and diabetic Zucker rats

3.1 Introduction

In humans, there is mounting evidence that the peripheral ECS is upregulated in obesity. This has been most conclusively demonstrated by several reports showing that plasma anandamide and 2-AG levels are increased in obese humans compared to lean (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Cote *et al.*, 2007). In adipose tissue, there are disagreements between studies as to whether various components of the ECS are up- or down-regulated in obesity. FAAH and CB₁ receptor mRNA levels have been most extensively reported (as described in detail in Section 1.5). In summary, CB₁ receptor mRNA (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Sarzani *et al.*, 2009; Bennetzen *et al.*, 2010) and protein (Bennetzen *et al.*, 2010) have been shown to be reduced in obese subjects compared to lean. Conversely, other research has shown CB₁ receptor mRNA to be increased in the subcutaneous adipose tissue of obese patients compared to lean (Pagano *et al.*, 2007). In contrast to these studies, another found no significant differences in CB₁ or CB₂ receptor mRNA between lean and obese groups (Murdolo *et al.*, 2007). In visceral adipose tissue, there are similar discrepancies. Some research has shown CB₁ receptor mRNA to be increased in obese patients compared to lean (Pagano *et al.*, 2007; Sarzani *et al.*, 2009; Bennetzen *et al.*, 2010), whilst another study showed that CB₁ receptor mRNA from lean and obese humans is not correlated with BMI (Lofgren *et al.*, 2007). Similarly, levels of FAAH mRNA in human adipose tissue have also been measured by multiple laboratories, with conflicting results. FAAH mRNA has been reported to be both increased (Murdolo *et al.*, 2007; Pagano *et al.*, 2007) and decreased (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Kempf *et al.*, 2007; Lofgren *et al.*, 2007) in the subcutaneous adipose tissue of obese compared to lean subjects.

It is important to note that in all of the above mentioned studies, the whole adipose tissue sample has been analysed, containing not only mature adipocytes, but other cells such as preadipocytes, fibroblasts, endothelial cells and macrophages (Trayhurn *et al.*, 2006). As a result, the effects of obesity on the ECS in mature adipocytes, the most metabolically active component of adipose tissue, remain under-reported. This is significant because it has recently been shown that the expression of CB₁ receptors in isolated mature adipocytes is lower than in the stromal-vascular fraction of adipose tissue (Bennetzen *et al.*, 2010). Additionally,

the reporting of mRNA in these studies leaves the question of how the activity of the ECS is affected by obesity in adipose tissue unanswered.

Several studies have indicated that the peripheral ECS may be dysregulated in type 2 diabetes, and that there may be crosstalk between insulin, glucose and the ECS. Some of the first evidence for this came from a study which showed increased concentrations of anandamide and 2-AG in blood plasma from humans with type 2 diabetes compared to matched controls of a similar BMI (Matias *et al.*, 2006). More recently, endocannabinoid levels have been reported in human subcutaneous adipose tissue. It was found that anandamide is increased and 2-AG is decreased in obese humans with type 2 diabetes compared to both lean humans and obese non-diabetic controls (Annuzzi *et al.*, 2010). FAAH has a crucial role in the regulation of anandamide levels and, given that peripheral anandamide levels may be increased in diabetes, the role of FAAH has been investigated in glycaemic homeostasis and diabetes. In one study, the euglycaemic-hyperinsulinaemic clamp technique was used to maintain hyperinsulinaemia in obese subjects and matched lean controls. This caused an upregulation of FAAH mRNA in the subcutaneous abdominal adipose tissue of lean subjects, but no change in the obese group, in which FAAH was already chronically upregulated. This suggests that hyperinsulinaemia alone may be sufficient to cause FAAH upregulation in adipose tissue (Murdolo *et al.*, 2007).

It is well established that weight loss in obese humans improves metabolic function, as measured by markers such as HOMA. A missense mutation in the FAAH gene has recently been associated with lack of improvements in these metabolic benefits, such that fasting serum insulin and glucose concentrations are not reduced after moderate weight loss (de Luis *et al.*, 2010a). Similarly, a study using FAAH knockout mice found that blood plasma insulin and glucose concentrations were increased compared to controls (Tourino *et al.*, 2010). It is not possible from these systemic studies to speculate on the role of FAAH specifically in adipocytes, or even in adipose tissue.

The CB₁ receptor has also been investigated in diabetes. In visceral adipose tissue, it has been reported that CB₁ receptor mRNA levels are not different between humans with and without diabetes (Lofgren *et al.*, 2007). However, CB₁ receptor signalling is thought to be important in glycaemic regulation, as rimonabant has hypoglycaemic properties and improves insulin sensitivity (Scheen *et al.*, 2006) and a CB₁ receptor

mutation has been associated with similar metabolic benefits (Bordicchia *et al.*, 2009). In addition to this, CB₁ receptor agonists have been shown to increase the rate of basal and insulin stimulated glucose uptake in cultured adipocytes (Gasperi *et al.*, 2007; Pagano *et al.*, 2007).

Taken together, these findings provide evidence for a role of the ECS in metabolic regulation and its dysregulation in diabetes, but as yet this has not been investigated in isolated mature adipocytes.

In this first study, three strains of Zucker rat were used to investigate healthy, obese (hyperinsulinaemic) and obese diabetic (hyperinsulinaemic and hyperglycaemic) states. The benefits of such animal studies are that each strain is well characterised and provides a discrete and homogenous population sample of the characteristic of interest. This is important given the high number of discrepancies observed between results reported on the ECS in adipose tissue in human obesity and diabetes, in which the samples represented a greater range of insulin sensitivities and percentage body fat. Importantly, the design of this study allowed the combined effects of type 2 diabetes and obesity to be compared against obesity in the absence of diabetes, as well as against lean, healthy rats.

Visceral adipose tissue is considerably more metabolically active than subcutaneous adipose tissue (for review see Wajchenberg, 2000), but as yet there is no conclusive evidence as to whether the activity of the ECS differs significantly between these depots. In normal weight subjects, CB₁ receptor expression has been reported to be higher in subcutaneous fat than visceral (Sarzani *et al.*, 2009; Bennetzen *et al.*, 2010), unchanged (Pagano *et al.*, 2007) or higher in visceral than subcutaneous (Blüher *et al.*, 2006). Conversely, in obese patients, CB₁ receptor mRNA was found to be elevated in the visceral adipose tissue depot compared to paired subcutaneous samples (Blüher *et al.*, 2006; Pagano *et al.*, 2007; Sarzani *et al.*, 2009) or not different (Bennetzen *et al.*, 2010). The two studies which have reported FAAH mRNA in adipose tissue are similarly inconsistent. In one study, FAAH mRNA was reported to be increased in visceral adipose tissue compared to subcutaneous in obese humans (Blüher *et al.*, 2006). However, another study showed no difference in FAAH mRNA between visceral and subcutaneous adipose tissue in lean or obese subjects (Pagano *et al.*, 2007). In this study, MGL mRNA was also measured and it was found that MGL was upregulated in subcutaneous adipose tissue compared to visceral adipose tissue in both lean and obese

groups (Pagano *et al.*, 2007). This finding is in keeping with another study which reported increased levels of 2-AG in visceral adipose tissue compared to subcutaneous in obese humans (Matias *et al.*, 2007). However, the catalytic activities of FAAH and MGL have not been reported or compared between different adipose tissue depots. Furthermore, none of these studies have reported on the ECS in isolated mature adipocytes as all have used whole adipose tissue. The importance of this has been highlighted recently in a study which reported both mRNA and protein levels for the CB₁ receptor. It was found that, in general, results obtained with both methods were similar. However, mRNA analysis showed a significant difference between lean and obese patients in visceral adipose tissue that was not apparent in CB₁ receptor protein levels (Bennetzen *et al.*, 2010).

3.2 Aims

In view of this background literature, the aim of this study was to investigate the activity of the ECS in isolated mature adipocytes in the Zucker rat models of obesity and diabetes. Initial plans for this study included measuring cannabinoid receptor activity, but preliminary experiments showed that the level of expression was too low to be detected using the methods available. In light of this, the study was designed to focus on FAAH and MGL activities. There were two key aims of the study, which were designed in order to address inconsistencies in the data published to date. The first of these was to compare the three strains of Zucker rat to determine whether FAAH or MGL activities in isolated mature adipocytes differ between healthy (lean), hyperinsulinaemic (obese) and diabetic (obese diabetic) rats. Secondly, the question of whether the ECS differs between visceral and subcutaneous adipose tissue depots was addressed by comparing enzyme activities in adipocytes from subcutaneous and two visceral adipose tissue sites.

3.3 Materials and Methods

3.3.1 Zucker rats and adipocyte preparation

Male Zucker rats as described in Section 2.1 were used. Adipose tissue was dissected from the subcutaneous abdominal, epididymal and abdominal visceral sites immediately after killing, stored at -80°C and processed as described in Section 2.2.

3.3.2 GTPyS binding assay

The GTPyS binding assay was performed with the membrane fraction of mature adipocytes pooled from all adipose tissue depots of several rats, as described in Section 2.6.1. The positive control was the membrane fraction of homogenised whole rat brain.

3.3.3 FAAH and MGL assays

The enzyme assays were performed as described in Section 2.5. Optimisation of the assays was achieved by using various dilutions of the homogenates, until activity was detectable, but produced no more than 10% substrate turnover.

3.3.4 Protein assay

The Bradford method was used to determine the protein concentration of each homogenate fraction, as described in Section 2.5.3.

3.3.5 Statistical analysis

GraphPad Prism software was used to analyse all of the results presented in this chapter. Comparisons between the three rat strains and three adipose tissue depots were made using one-way ANOVA and Bonferroni's *post hoc* test. The correlation studies were performed using linear regression and the Pearson correlation coefficient is reported. For all tests, $P < 0.05$ was deemed statistically significant.

3.4 Results

3.4.1 Characteristics of Zucker rats

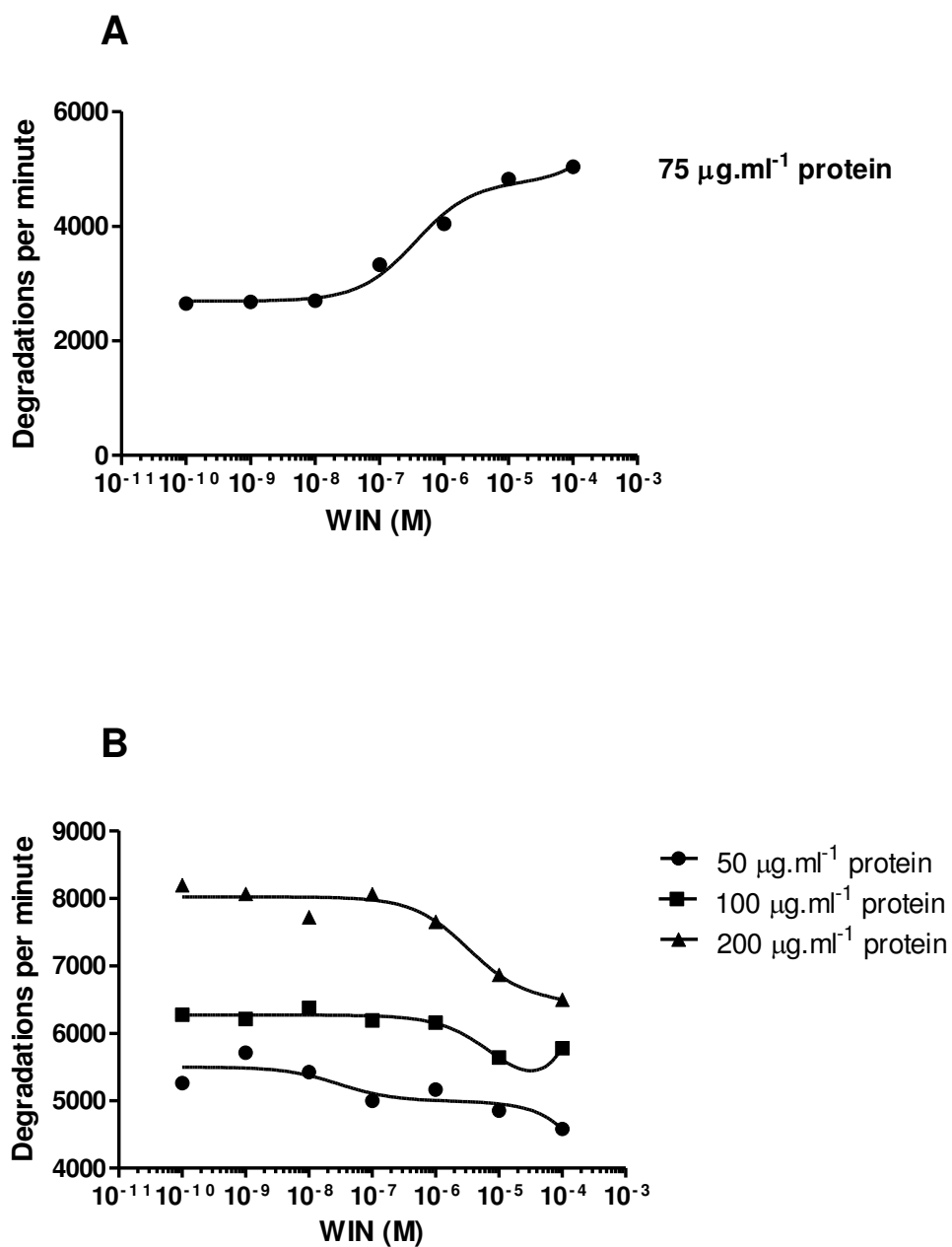
In this study 22 Zucker rats were used. These included lean, obese and obese diabetic rats, and the mean body weight and blood glucose concentration for each group is given in Table 3.1. Blood glucose data are missing for two of the obese diabetic rats, and weights were not recorded for two of the obese rats.

	Body weight (g)	Non-fasting blood glucose (mmol.L ⁻¹)
Lean	288.5±6.2	6.6±0.3
Obese	362.7±6.7	10.2±0.6
Obese diabetic	324.5±6.7	18.3±1.7

Table 3.1 Body weight and blood glucose concentrations of Zucker rats

3.4.2 Cannabinoid receptor assays

The GTPγS binding assay (described in Section 2.6.1) measures the amount of [³⁵S]GTPγS bound to the Gα subunit of the G protein, and therefore the relative level of G protein activation caused by coupled receptor agonists (Harrison and Traynor, 2003). In this study, this assay produced a typical dose response curve to WIN-55,212 using a positive control sample (rat brain membranes, Figure 3.1A). However, no binding was achieved using the rat adipocyte membrane fraction at protein concentrations of 50, 100 or 200 µg.ml⁻¹ (*n*=6, Figure 3.1B), indicating that CB₁ and CB₂ receptor activity was not detectable in the rat mature adipocyte membranes.

Figure 3.1 GTP γ S binding

GTP γ S binding in positive control (rat brain, A) and three concentrations of rat adipocyte membranes (B).

3.4.3 Enzyme activity

Anandamide hydrolysis in the rat adipocytes was suppressed by the FAAH inhibitor URB597 at 1 μ M (Figure 3.2A). FAAH activity was present in the membrane fraction of the homogenised adipocytes, but was not detected in the cytosolic fraction (Figure 3.2B). Similar results for the distribution of FAAH have been reported previously (McKinney and Cravatt, 2005).

The majority of adipocyte MGL activity was detected in the cytosolic fraction (Figure 3.2D), with only a minor amount in the membrane fraction (Figure 3.2C). MAFP (1 μ M), a non-specific MGL inhibitor, completely suppressed 2-OG hydrolysis (Figure 3.2C).

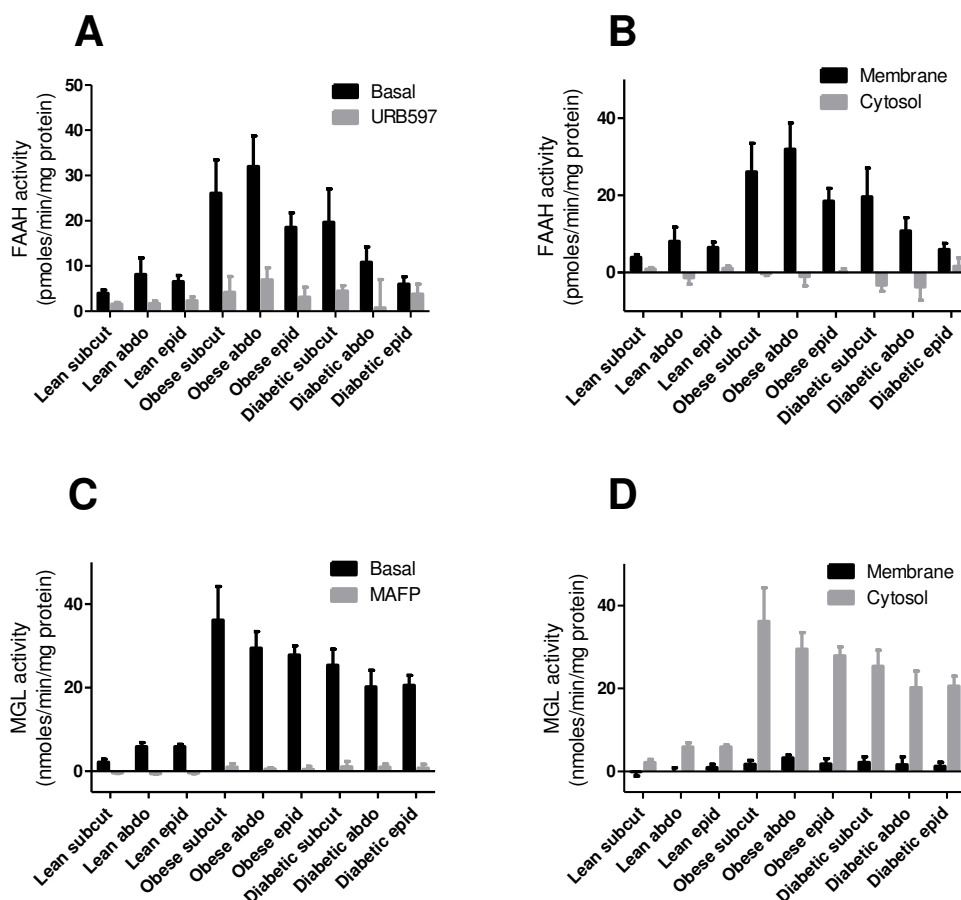


Figure 3.2 Inhibition of FAAH and MGL

FAAH activity in the presence of URB597 (1 μ M) or vehicle in the membrane fraction of adipocyte homogenates (A). MGL activity in the presence of MAFP (1 μ M) or vehicle in the cytosolic fraction of adipocyte homogenates (C). FAAH (B) or MGL (D) activity in the membrane and cytosolic fractions of adipocyte homogenates. Data are given as means with error bars representing S.E.M.

3.4.4 Enzyme activity in three rat strains

The obese rats showed significantly higher FAAH activity than the lean or obese diabetic rats in the abdominal ($P<0.05$, Figure 3.3C) and epididymal ($P<0.01$, Figure 3.3E) adipocytes. In the subcutaneous adipocytes, there was no significant difference between the lean, obese and diabetic rats (Figure 3.3A).

The obese and obese diabetic rats had elevated MGL activity compared to the lean rats in all adipocytes. In the subcutaneous adipocytes, MGL activity was higher in the obese rats than the lean ($P<0.01$, Figure 3.3B) and in the abdominal and epididymal adipocytes this relationship reached a higher level of significance ($P<0.001$, Figure 3.3D,F). MGL activity in the diabetic rats was higher than in the lean rats in subcutaneous ($P<0.05$, Figure 3.3B), abdominal ($P<0.05$, Figure 3.3D) or epididymal ($P<0.001$, Figure 3.3F) adipocytes.

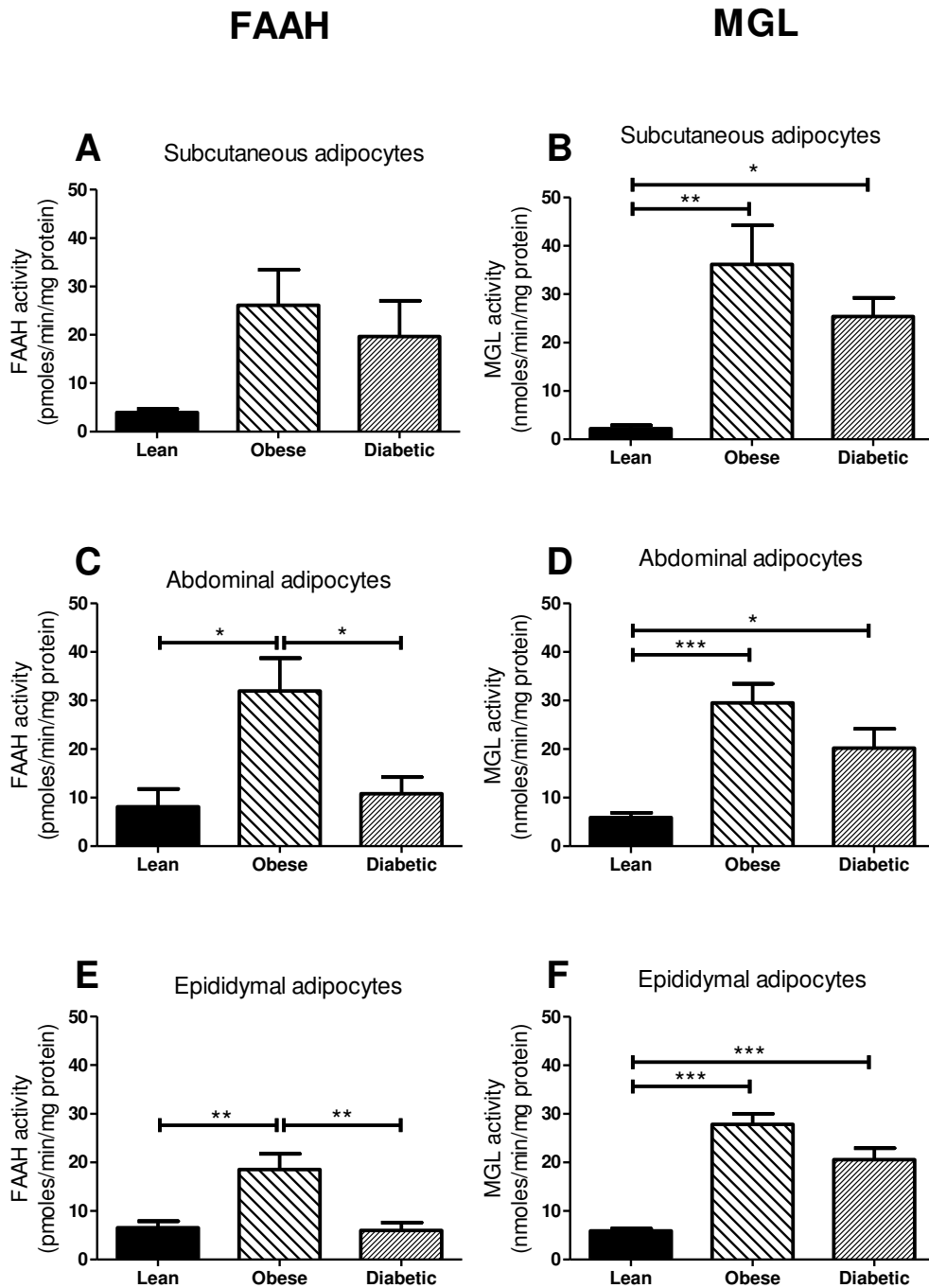


Figure 3.3 FAAH and MGL in three rat strains

FAAH and MGL activities in mature adipocytes isolated from subcutaneous (A,B), abdominal (C,D) and epididymal (E,F) adipose depots in lean, obese and diabetic Zucker rats. Data presented here are the same as Figure 3.5, but displayed to allow comparison between the three rat strains. $n = 6$ for control rats and $n = 8$ for obese and obese diabetic rats. Data are given as means, with error bars representing S.E.M., and were analysed by one-way ANOVA and Bonferroni's *post hoc* test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$).

3.4.5 FAAH activity and body mass

Correlation studies were performed between FAAH activity in the three rat strains and total body mass. In the subcutaneous adipocytes, there was no relationship between FAAH activity and body mass (Figure 3.4A), but in the abdominal and epididymal adipocytes FAAH activity correlated positively with body mass ($r=0.54$, $P<0.05$; $r=0.49$, $P<0.05$, Figure 3.4C,E).

3.4.6 MGL activity and body mass

A positive relationship was identified between MGL activity and body mass in adipocytes from all three adipose tissue depots (subcutaneous $r=0.72$, $P<0.001$, Figure 3.4B; abdominal $r=0.63$, $P<0.01$, Figure 3.4D; epididymal $r=0.80$, $P<0.001$, Figure 3.4F).

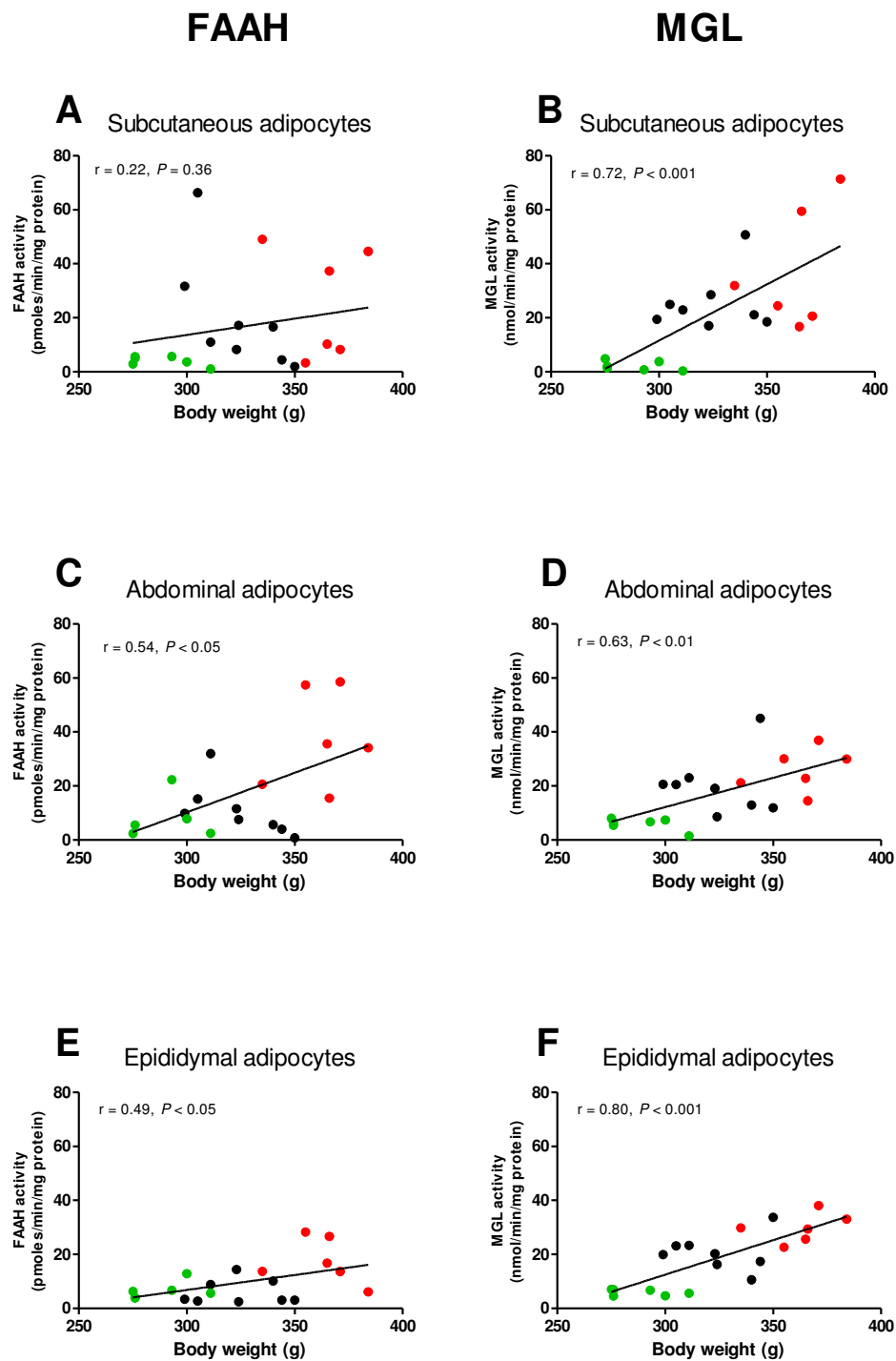


Figure 3.4 FAAH and MGL with body mass

Correlative studies between the total body mass of each rat and enzyme activity in mature adipocytes isolated from each adipose tissue depot. Data are presented for subcutaneous (A,B), abdominal (C,D) and epididymal (E,F) adipocytes and the Pearson correlation coefficient is reported. Key: green, lean rats; red, obese rats; black, obese diabetic rats.

3.4.7 Enzyme activity between adipose tissue depots

In the lean Zucker rats, FAAH activity did not differ between the subcutaneous, abdominal and epididymal adipose tissue depots (3.9 ± 0.7 , 8.1 ± 3.7 , 6.5 ± 1.3 pmoles.min⁻¹.mg protein⁻¹ respectively, Figure 3.5A). However, in the lean rats, MGL activity was significantly lower in the subcutaneous adipocytes than in the abdominal and epididymal adipocytes (2.2 ± 0.7 , 5.9 ± 1.0 , 5.9 ± 0.5 nmoles.min⁻¹.mg protein⁻¹ respectively, $P < 0.01$, Figure 3.5B).

In the obese Zucker rats, FAAH activity was not different between the three adipose tissue depots (subcutaneous: 26.1 ± 7.4 , abdominal: 32.0 ± 6.7 and epididymal: 18.5 ± 3.2 pmoles.min⁻¹.mg protein⁻¹, Figure 3.5C). In contrast to the results for the lean rats, MGL activity did not differ between the subcutaneous, abdominal and epididymal sites (36.2 ± 8.1 , 29.5 ± 3.9 , 27.8 ± 2.2 nmoles.min⁻¹.mg protein⁻¹ respectively, Figure 3.5D).

In the obese diabetic rats the results were the same as the obese rats in that FAAH activity in mature adipocytes did not differ between the three adipose tissue depots (19.6 ± 7.4 , 10.8 ± 3.4 , 6.0 ± 1.6 pmoles.min⁻¹.mg protein⁻¹, Figure 3.5E) and neither did MGL activity (25.4 ± 3.8 , 20.2 ± 4.0 , 20.6 ± 2.4 nmoles.min⁻¹.mg protein⁻¹, Figure 3.5F).

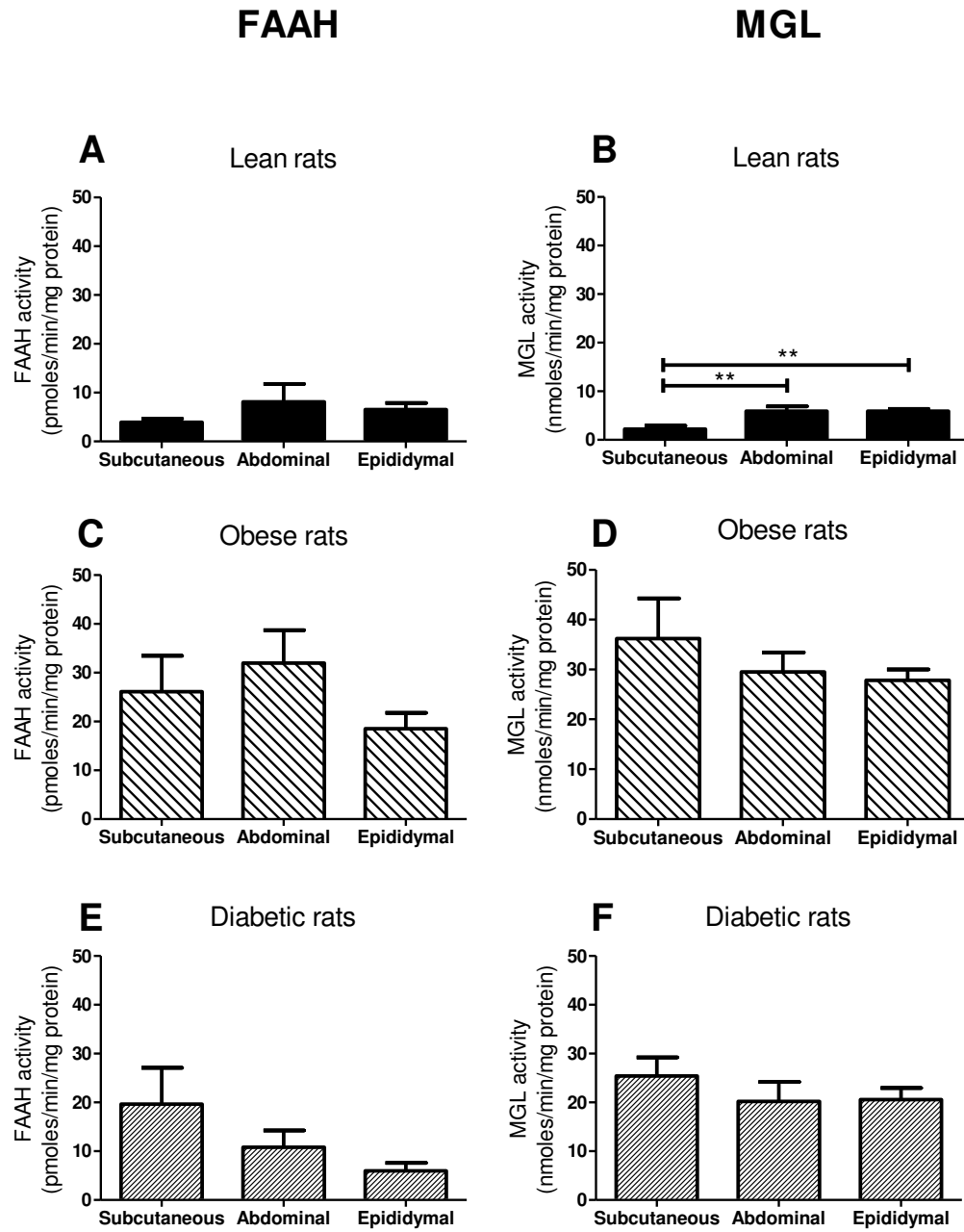


Figure 3.5 FAAH and MGL in three adipose tissue depots

FAAH and MGL activities in mature adipocytes isolated from three adipose depots in control (A,B), obese (C,D) and obese diabetic (E,F) Zucker rats. $n = 6$ for control rats and $n = 8$ for obese and obese diabetic rats. The data are the same as those presented in Figure 3.3, but are arranged in this figure to allow comparison between the three adipose tissue depots. Data are given as means, with error bars representing S.E.M., and were analysed by one-way ANOVA and Bonferroni's *post hoc* test (** $P < 0.01$).

3.4.8 Enzyme activity and blood glucose

Neither FAAH nor MGL (Figure 3.6) activity in adipocytes correlated with non-fasting blood glucose concentration in any of the rats or adipose tissue depots investigated.

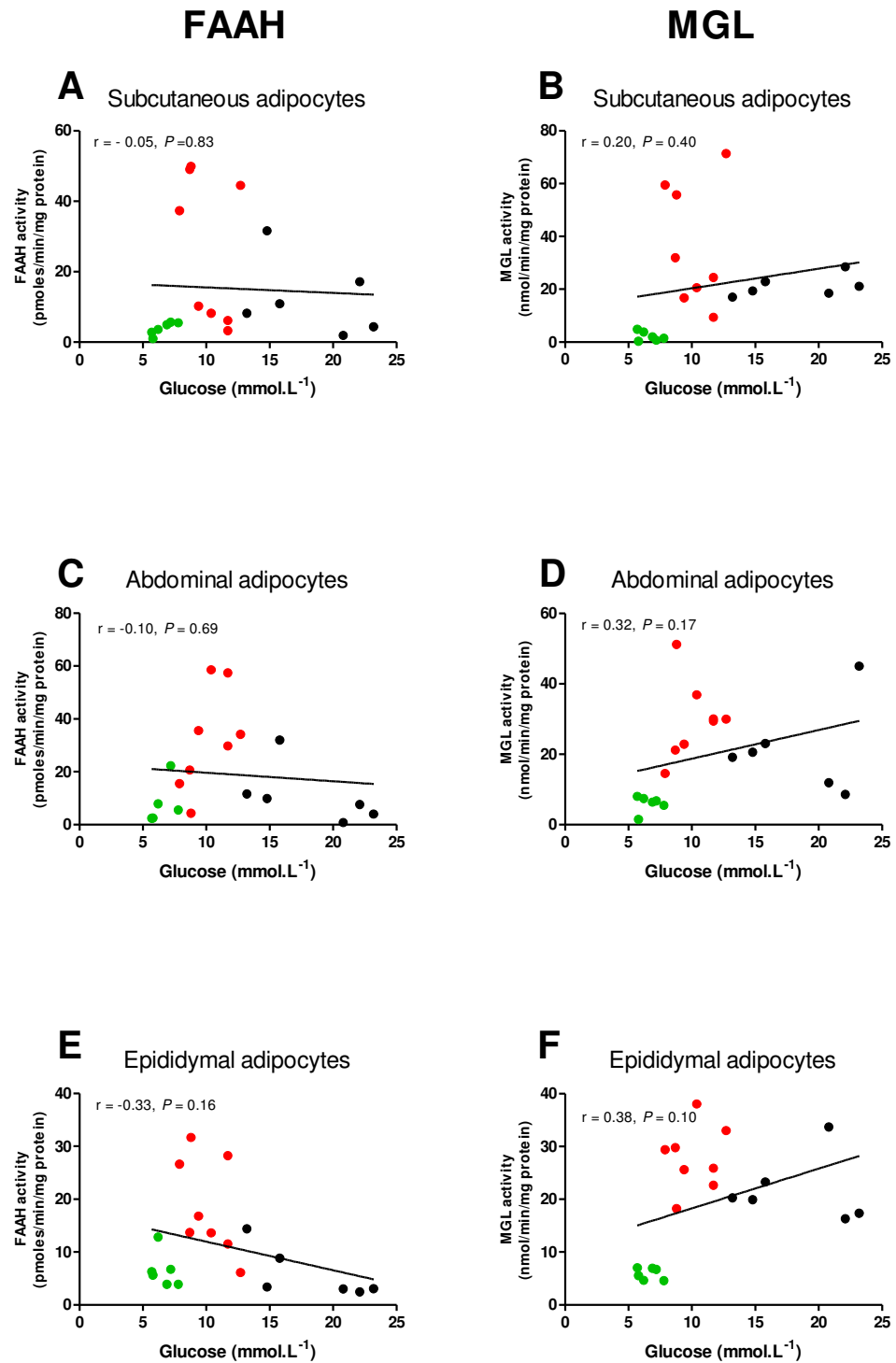


Figure 3.6 FAAH and MGL with blood glucose

Correlative studies between blood glucose concentration and enzyme activity in mature adipocytes isolated from each adipose tissue depot. Data are presented for subcutaneous (A,B), abdominal (C,D) and epididymal (E,F) adipocytes and the Pearson correlation coefficient is reported. Key: green, lean rats; red, obese rats; black, obese diabetic rats.

3.5 Discussion

Over recent years, there has been increasing speculation and evidence that the peripheral ECS is upregulated in human and animal obesity, albeit with many conflicts in the published data. In view of this, the primary aim of this study was to re-examine this topic in a novel manner by determining whether the activities (as opposed to mRNA) of the two major catabolic enzymes of the ECS, FAAH and MGL, are altered in mature adipocytes (as opposed to the whole adipose tissue) in a Zucker rat model of obesity. There have also been reports that the ECS may be affected by metabolic dysregulation or upregulated in type 2 diabetes. Therefore, the second aim of this study was to establish whether enzyme activities are affected by hyperinsulinaemia in obese rats, or diabetes in obese diabetic rats, compared to the healthy lean controls. Finally, given the discrepancies reported for FAAH and MGL mRNA levels in visceral and subcutaneous adipose tissue, the activities of these enzymes in isolated adipocytes were compared between three different adipose tissue depots in each rat strain.

The main findings of this study were that FAAH activity correlated with total body mass in the visceral adipocytes, and MGL activity correlated with total body mass in adipocytes from all three depots. Direct comparisons between the three rat strains showed that the obese rats tended to have increased FAAH activity compared to the lean and obese diabetic rats. Furthermore, both the obese and obese diabetic rats had higher MGL activity than the lean rats. Together, this supports the theory that the ECS is dysregulated in obesity. In general, FAAH and MGL activities in isolated mature adipocytes were not different between the three adipose tissue depots analysed.

Previous studies have demonstrated that CB₁ and CB₂ receptor proteins are present and functional in human mature adipocytes. This has been shown using various methods such as immunostaining, Western blotting and measuring cAMP production in the presence of cannabinoid receptor agonists and antagonists (Engeli *et al.*, 2005; Roche *et al.*, 2006). CB₁ receptor mRNA has also been detected in rat adipocytes (Karaliota *et al.*, 2009) and in the adipose tissue of obese Zucker rats (Bensaid *et al.*, 2003). In human studies, CB₁ receptor mRNA levels in subcutaneous adipose tissue have been reported to be altered in obesity, with some studies showing downregulation in obesity (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Sarzani *et al.*, 2009; Bennetzen *et al.*, 2010) and others showing upregulation or no change (Lofgren *et al.*, 2007; Murdolo *et al.*,

2007; Pagano *et al.*, 2007). However, whether the activity of the cannabinoid receptors is altered in obesity has not been reported. An aim of this study was to measure cannabinoid receptor activity in mature adipocytes from lean and obese Zucker rats using the GTP γ S binding assay. This assay worked as expected using a positive control (Zucker rat whole brain membranes), giving a sigmoidal dose response curve to WIN-55,212. WIN-55,212 is a non-specific CB₁ and CB₂ receptor agonist, so without the use of specific antagonists, the activity reported here for the rat brain control is total cannabinoid receptor activity. In the results presented here for rat mature adipocytes, a dose response curve was not seen at the three protein concentrations and previous work in this laboratory has shown that the assay is not reliable at protein concentrations outside of this range. This suggests that in these adipocytes there is no appreciable CB₁ or CB₂ receptor activity and endocannabinoids may therefore signal through alternative receptors, such as the TRPs and PPARs in Zucker rat adipocytes, potentially increasing rates of lipolysis and differentiation. Additionally, the endocannabinoids may act on other cells in adipose tissue more than mature adipocytes. In human samples, CB₁ receptor mRNA is markedly lower in whole adipose tissue than in brain (Engeli *et al.*, 2005). Additionally, a recent study showed that CB₁ receptor mRNA levels are higher in the stromal-vascular fraction of human subcutaneous adipose tissue than in isolated mature adipocytes (Bennetzen *et al.*, 2010). This may explain why cannabinoid receptor activity was not detected using the GTP γ S assay in the mature adipocytes. In the study which showed cannabinoid receptor activity (via cAMP reduction), the isolated adipocytes were cultured for 18 hours prior to the activity assays (Roche *et al.*, 2006), whereas in the current study, the mature adipocytes were stored at -80°C, and to date the effects of cell culture on the ECS have not been well characterised. The findings of this study, which differ from studies showing that cannabinoid receptor mRNA and protein are present, highlight the importance of measuring protein activity. However, to do this in isolated mature adipocytes, alternative assays may need to be developed.

Given these issues with measuring cannabinoid receptor activity in mature adipocytes, future studies in this thesis were focussed on the activities of FAAH and MGL. The mRNA levels of MGL, and particularly FAAH, in adipose tissue have been relatively extensively reported in studies investigating the ECS in obesity and diabetes. However, the

activities of FAAH and MGL have not been reported in these comparison studies and it is therefore unknown whether alterations in mRNA expression relate to changes in final activities of the enzymes. Additionally, the effects of obesity and diabetes on the ECS enzymes in isolated mature adipocytes, rather than whole adipose tissue, have not been reported. Enzyme activity assays in mature adipocyte homogenates were used in this study to address these issues. These assays, which measure the rate of hydrolysis of anandamide and 2-OG, have been described and optimised in several published studies (Dinh *et al.*, 2002b; Wilson *et al.*, 2003; Boldrup *et al.*, 2004; Ghafouri *et al.*, 2004). As all labelled products are detected, enzyme inhibitors are used to indicate the percentage of this total hydrolysis that can be attributed to FAAH or MGL. URB597 is a specific FAAH inhibitor which inhibits anandamide hydrolysis in rat and human tissues with IC_{50} s in the low nanomolar range (reviewed by Piomelli *et al.*, 2006). *In vivo* URB597 has been shown to increase the concentrations of several fatty acid ethanolamide substrates of FAAH, and does not appreciably affect MGL activity (Piomelli *et al.*, 2006). The use of URB597 in this study indicates that almost 80% of the anandamide hydrolysis reported in isolated adipocytes is due to FAAH activity. The inhibitor used in the MGL assay, MAFP, is less specific in its binding than URB597. It has been shown to inhibit both MGL and FAAH and to bind to the CB₁ receptor (Martin *et al.*, 2000; Ho and Hillard, 2005). The use of MAFP in the 2-OG hydrolysis assay completely suppressed hydrolysis. The first reported MGL activity experiments in rat epididymal adipose tissue homogenates showed that approximately half of the total MGL activity in whole adipose tissue homogenates was in the supernatant fraction, with the remainder of the activity in the fat cake and the membrane pellet (Tornqvist and Belfrage, 1976). The proportion of 2-OG hydrolytic activity detected in the supernatant fraction of the mature adipocyte homogenates used in the current study was significantly greater than 50%. Importantly, the study showing significant MGL activity in the adipose tissue particulate fraction used a longer centrifugation time at greater force (Tornqvist and Belfrage, 1976), and it is possible that this reduced the concentration of MGL in the supernatant fraction. In addition to this, MGL is found in lipid aggregates which contain proteins. These may be artefacts from the homogenisation process (Tornqvist and Belfrage, 1976) and differences in the homogenisation protocols used between studies may also account for some of the observed differences in MGL activity distribution. It should also be

noted that in our study only mature adipocytes were used rather than whole adipose tissue.

As previously described in detail, changes in the peripheral ECS in obesity have been widely reported in human studies. Circulating endocannabinoid levels are generally reported to be elevated, but studies in adipose tissue have not been conclusive. FAAH mRNA has been reported to be up and downregulated. In order to increase the data available on FAAH regulation in the most metabolically active component of adipose tissue, this study focussed specifically on FAAH activity in isolated mature adipocytes. It was found that in visceral adipocytes, FAAH activity correlates with total body mass, and in direct comparisons of the three Zucker rat strains, FAAH activity was higher in the obese rats than both the lean and obese diabetic rats in adipocytes from the two visceral adipose tissue depots. Another study using obese animals showed that FAAH protein levels in mice with diet-induced obesity were increased in subcutaneous adipose tissue. However, in this study, FAAH protein levels were not affected in the visceral adipose tissue (Starowicz *et al.*, 2008). This finding is similar to that of a human study which found no difference in FAAH mRNA in visceral adipose tissue between lean and obese subjects, but an increase in expression in abdominal subcutaneous adipose tissue (Pagano *et al.*, 2007). By contrast to our results, another human study reported that FAAH mRNA is decreased in both visceral and subcutaneous adipose tissue in obesity (Blüher *et al.*, 2006).

Whilst FAAH mRNA levels in adipose tissue have been investigated in several published studies, MGL has not, and its expression remains relatively under-reported. It has been shown in one study that MGL mRNA levels in both subcutaneous and visceral adipose tissue are increased in obesity (Pagano *et al.*, 2007). The results presented in this chapter show that MGL activity correlates with total body weight in adipocytes from all three adipose tissue depots and MGL activity is greater in the obese and obese diabetic rats than in the healthy lean rats. These findings appear to be in agreement with the study that showed MGL mRNA to be increased in abdominal subcutaneous and visceral adipose tissue in obese humans compared to lean (Pagano *et al.*, 2007). An increase in MGL mRNA in visceral adipose tissue has also been reported in mice with diet-induced obesity (D'eon *et al.*, 2008). Increased MGL activity in mature adipocytes in obesity may explain why the concentration of 2-AG in the subcutaneous

adipose tissue of obese Zucker rats was found to be lower than in lean rats (Izzo *et al.*, 2009).

These results indicate that, in general, the activities of the major catabolic enzymes of the ECS, FAAH and MGL, are upregulated in mature adipocytes in obesity. This finding in Zucker rats supports the notion that regulation of the peripheral ECS is altered in obesity.

There is some evidence that the ECS is dysregulated in diabetes and may be under the regulation of insulin (Di Marzo *et al.*, 2009). Although there are few published studies in this area of research, one study reported that circulating endocannabinoid levels are increased in type 2 diabetes (Matias *et al.*, 2006). Levels of anandamide have also been found to be increased in the subcutaneous adipose tissue of obese diabetic humans compared to obese non-diabetics (Annuzzi *et al.*, 2010). In another study, in lean humans, exogenous insulin has been shown to increase FAAH mRNA in subcutaneous adipose tissue (Murdolo *et al.*, 2007). These studies suggest that the ECS in adipose tissue may be altered in diabetes, or be under the regulation of insulin. With the studies published to date in adipose tissue biopsies, it is not possible to conclude whether these changes occur in mature adipocytes. In the study presented here, mature adipocytes were isolated from lean, obese and obese diabetic rats in order to ascertain whether FAAH activity is affected by hyperinsulinaemia (obese rats) or hyperinsulinaemia with hyperglycaemia (insulin resistant, diabetic rats). The results show that in subcutaneous adipocytes, there is no difference in FAAH activity between lean, obese and obese diabetic rats. By contrast, in mature adipocytes from both of the visceral adipose tissue depots tested, FAAH activity is higher in the obese rats than both the healthy and obese diabetic rats. Furthermore, there is no difference in FAAH activity between the lean and obese diabetic rats. In some respects, these findings appear to be in agreement with a human study which reported FAAH mRNA in subcutaneous adipose tissue to be regulated by insulin. In this study, FAAH mRNA was increased in obesity, as seen here with activity, and differences in response to intravenous insulin infusion were observed between lean and obese subjects. In the lean subjects, FAAH mRNA was increased, but in the obese humans FAAH mRNA levels did not change in response to insulin (Murdolo *et al.*, 2007). These findings show that in an obese, pre-diabetic, moderately insulin resistant state, FAAH is chronically upregulated by insulin, but not sensitive to acute upregulation by insulin. This may be one explanation as to why in

the completely insulin resistant, diabetic rats used in our study, FAAH activity was found to be at lean baseline levels despite hyperinsulinaemia. Another study, which measured endocannabinoid levels in subcutaneous adipose tissue, is also relevant to our findings. It was shown that anandamide is higher in obese diabetic humans than in obese non-diabetics (Annuzzi *et al.*, 2010). Although this was shown in subcutaneous adipose tissue, it is otherwise in line with the results reported here in visceral adipocytes showing increased FAAH activity in obese but not obese diabetic rats. The novel results reported in this chapter support the notion that FAAH regulation is altered in obese diabetic compared to obese rats in visceral mature adipocytes, possibly by insulin.

In these studies, positive results have been reported for insulin rather than glucose. In agreement with this, the results of the current study show no correlations between blood glucose concentrations and FAAH or MGL activity in adipocytes from any of the adipose tissue depots sampled. A study into MGL activity in rat adipocytes found that activity did not differ between fasted and fed rats (Tornqvist *et al.*, 1978), which is in keeping with the results presented here showing that the glucose concentration at the time of sampling does not correlate with MGL activity. Overall, these results do not suggest that acute hyperglycaemia affects FAAH or MGL activity in mature adipocytes.

In addition to increased blood plasma concentrations of 2-AG in humans with type 2 diabetes (Matias *et al.*, 2006), a decrease in 2-AG in subcutaneous adipose tissue has been reported in obese diabetic humans (Annuzzi *et al.*, 2010). Given this evidence for dysregulated 2-AG levels, it was considered important to determine whether MGL activity in mature adipocytes is altered in the Zucker rat models of obesity and type 2 diabetes. The results of this study show that MGL activity is increased in the obese and obese diabetic rats compared to the lean in mature adipocytes from all adipose tissue depots. An interesting difference to the FAAH results is that MGL activity is not different between the obese and obese diabetic rats. Finally, MGL activity in the adipocytes did not correlate with blood glucose concentration. These results are in keeping with the human study which showed 2-AG levels to be decreased in subcutaneous adipose tissue in type 2 diabetes (Annuzzi *et al.*, 2010). An increase in MGL activity in obesity and type 2 diabetes may reduce 2-AG mediated signalling in adipocytes at receptors such as PPAR γ and CB $_1$, potentially

further reducing insulin sensitivity (Rockwell *et al.*, 2006; Motaghedi and McGraw, 2008).

The subcutaneous and visceral adipose tissue depots are often considered to be metabolically distinct, with excess visceral adipose tissue conferring a greater risk of development of type 2 diabetes and other metabolic disorders than excess subcutaneous adipose tissue (Bray *et al.*, 2008; Taksali *et al.*, 2008). In two human studies, the mRNA levels of FAAH have been compared between subcutaneous and visceral adipose tissue, but the findings are inconclusive. In the first, FAAH was found to be upregulated in visceral adipose tissue, regardless of BMI (Blüher *et al.*, 2006), whereas the second showed no difference between visceral and subcutaneous adipose tissue (Pagano *et al.*, 2007). In a study using obese Zucker rats, FAAH activity was found to be higher in the subcutaneous than visceral adipose tissue (Batetta *et al.*, 2009), but this has not been investigated in isolated mature adipocytes. The results of the current study show that in the lean healthy rats there is no difference in FAAH activity between the subcutaneous and visceral (abdominal and epididymal) adipocytes. This was also found in the obese and obese diabetic rats. These findings are in keeping with the human study which showed no difference in FAAH mRNA levels between visceral and subcutaneous adipose tissue, even in obesity (Pagano *et al.*, 2007). By contrast, the relative activities of FAAH reported here do not reflect the differences in mRNA between subcutaneous and visceral adipose tissue reported by Blüher *et al.* (2007). Importantly, the results presented here are also at odds with the study which reported increased FAAH activity in subcutaneous versus visceral adipose tissue in obese Zucker rats (Batetta *et al.*, 2009). Given the similarities between this latter study and the work presented here, one of the most likely explanations for this difference is that in the current study isolated mature adipocytes were used rather than whole adipose tissue. It has been shown that macrophages have significant FAAH expression (Sun *et al.*, 2005), but the FAAH activity of other cells of the stromal-vascular fraction of adipose tissue have not been reported. Therefore, differences in FAAH activity in the stromal-vascular cells between visceral and subcutaneous sites could account for the apparent discrepancy. The results given here indicate that the rate of anandamide degradation in mature adipocytes is not different between the three adipose tissue depots, and that this balance is not affected by obesity or diabetes.

MGL is known to have an important role in lipid metabolism, but as yet its activity has not been investigated in mature adipocytes from visceral and subcutaneous adipose tissue depots. Given the essential role of MGL in 2-AG catabolism, and therefore in ECS signalling, its activity between depots was investigated in this study. The results show that in the lean healthy rats MGL activity is increased in adipocytes from both of the visceral adipose tissue depots compared to the subcutaneous adipocytes. In the obese and obese diabetic rats this difference is lost, and MGL activity is similar in adipocytes from all three depots, as was found with FAAH activity. This finding is at odds with the only study which has compared MGL mRNA between depots, as this human study showed increased MGL expression in subcutaneous compared to visceral adipose tissue (Pagano *et al.*, 2007). As explained previously, this may be due to the use of isolated mature adipocytes in our study, or the analysis of activity rather than mRNA. It is interesting to note the differences observed here between the lean healthy rats and the obese rats. In the lean rats with normal metabolic physiology, MGL activity is higher in the visceral adipocytes than the subcutaneous, as might be expected given that visceral adipose tissue is more metabolically active (Wajchenberg, 2000). However, in both of the obese Zucker rat strains, with disordered metabolic homeostasis, the level of MGL activity is higher and not different between visceral and subcutaneous adipocytes. This may be an indication that in obesity, subcutaneous adipocytes are recruited to assist in lipolysis that predominantly occurs in visceral adipocytes in the healthy state. This increase in MGL activity in subcutaneous adipocytes in obesity and diabetes might reduce 2-AG signalling at both cell surface and intracellular receptors.

In summary, despite evidence of dysregulation of the ECS in adipose tissue in obesity and type 2 diabetes, current data comparing mRNA levels of ECS components in adipose tissue are inconclusive. In light of this, FAAH and MGL activities were assayed in isolated mature adipocytes from lean, obese and obese diabetic Zucker rats. The major findings were that FAAH and MGL activities increased with body mass and there were differences between lean, obese and diabetic rats. FAAH activity was increased in obese rats, and MGL activity was found to be increased in obese and obese diabetic rats. Furthermore, in general, the enzyme activities did not differ in adipocytes from different adipose tissue depots. These changes have the potential to significantly modulate the

signalling of anandamide and 2-AG. Having identified these relationships in FAAH and MGL activities in Zucker rat models of obesity and diabetes, in the next chapter a study to investigate whether these enzymes are also altered in adipocytes in human obesity is presented.

4. Enzyme activities in subcutaneous human mature adipocytes

4.1 Introduction

One of the major findings of Chapter 3 was that FAAH and MGL activities are increased in adipocytes from obese rats. This is consistent with the notion that overall ECS tone is increased in human obesity. Some of the studies which led to this hypothesis showed that plasma levels of anandamide (Engeli *et al.*, 2005) and 2-AG (Blüher *et al.*, 2006; Cote *et al.*, 2007) are increased in obesity. Circulating 2-AG levels have also been shown to correlate with waist circumference (Engeli *et al.*, 2005) and, accordingly, more in depth analysis revealed that the most significant rise in plasma 2-AG occurs in those with visceral obesity (Blüher *et al.*, 2006; Cote *et al.*, 2007). Additionally, weight loss in obese men has been shown to reduce plasma levels of both AEA and 2-AG (Di Marzo *et al.*, 2008). 2-AG has also been reported to be increased in visceral adipose tissue in human obesity (Matias *et al.*, 2006). Despite the reasonably consistent findings in circulating endocannabinoid levels between different studies, the relative expression levels of other components of the ECS in adipose tissue in obese compared to lean humans remain debated.

As described in Section 1.5.5 and Chapter 3, levels of FAAH mRNA in human adipose tissue have been measured by multiple laboratories and conflicting findings have been reported. In some studies, FAAH mRNA is reported to be higher in the subcutaneous adipose tissue of obese compared to lean subjects (Murdolo *et al.*, 2007; Pagano *et al.*, 2007; Bennetzen *et al.*, 2011), whereas other studies report FAAH mRNA to be decreased (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Lofgren *et al.*, 2007). There are no obvious reasons as to why discrepancies have been reported with regard to FAAH expression levels in adipose tissue in obesity. The techniques used in these studies appear to have been similar, as do the subjects sampled, although females are represented more than males in the studies showing FAAH to be downregulated in obesity, and males are a larger proportion of the results showing FAAH to be upregulated. A further connection between FAAH and obesity has been identified via a missense mutation in the FAAH gene, which reduces the protein expression and activity of FAAH and occurs in 3.6-10.8% of the population (depending on ethnicity) (Sipe *et al.*, 2005). This mutation has been associated with obesity, as have some variants in the promoter section of the gene (Harismendy *et al.*, 2010).

The studies described above have compared discrete groups of lean and obese subjects, with overweight (BMI 25-30 kg.m⁻²) humans excluded. In addition to this, all of the published studies on FAAH in adipose tissue in obesity have reported mRNA levels of FAAH without reference to final protein levels or activity. FAAH activity has been reported in human adipose tissue, (Spoto *et al.*, 2006) but not in a comparative study, and not in isolated mature adipocytes. In order to increase our understanding of the role of FAAH in human adipocytes, and whether this alters with BMI, it is important to investigate FAAH activity, and thus endocannabinoid degradation, in isolated adipocytes. This is also important given that previous studies have investigated the ECS in whole adipose tissue rather than in mature adipocytes.

MGL expression in human adipose tissue has not yet been extensively investigated with regard to obesity. In one study, MGL mRNA in subcutaneous adipose tissue was compared between distinct cohorts of lean and obese humans. It was found that obesity had no effect on MGL expression in abdominal subcutaneous adipose tissue (Lofgren *et al.*, 2007). However, in another study MGL mRNA was found to be upregulated in the abdominal subcutaneous adipose tissue of obese subjects compared to lean (Pagano *et al.*, 2007). In agreement with this study, the results of Chapter 3 showed that MGL activity is increased in abdominal subcutaneous adipocytes in obese rats compared to lean. As plasma 2-AG is increased in obesity (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Cote *et al.*, 2007), and there is speculation that 2-AG secretion from adipocytes may contribute to this, it is important to establish the effects of obesity on MGL activity in mature adipocytes in humans.

Given that the majority of the studies described here have compared lean and obese subjects, there are remarkably few data on overweight humans (BMI 25-30 kg.m⁻²) and the ECS in adipose tissue. It was decided that overweight humans would be included in the following study in order to improve our understanding of the ECS in the pre-obese state, and possibly indicate whether ECS dysregulation occurs prior to overt obesity. In addition to this, although in the majority of the healthy non-athletic population, BMI correlates with overall body fat percentage (Deurenberg *et al.*, 1991), the two measurements give different information. This has been revealed in some studies into the ECS in obesity. In some cases there is a significant relationship between the ECS and BMI, but not body fat percentage or other adiposity markers, and

sometimes relationships are observed with body fat and its distribution, but not BMI (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Cote *et al.*, 2007). As BMI alone is not an accurate measure of adiposity and body fat distribution, and does not take into account variability between humans of the same BMI, anthropometric estimations of adiposity (circumferences and skinfold thicknesses) were used in the following study.

4.2 Aims

In light of this background literature, the primary aim of the current study was to investigate the activity of FAAH and MGL in subcutaneous mature adipocytes from healthy humans representing a continuous range of BMIs. Assays were undertaken in mature adipocytes isolated from human subcutaneous adipose tissue in order to exclude interference from other cells in adipose tissue, such as preadipocytes or immune cells (other studies measured enzyme expression in the entire adipose tissue sample (Engeli *et al.*, 2005, Blüher *et al.*, 2006, Spoto *et al.*, 2006, Lofgren *et al.*, 2007, Murdolo *et al.*, 2007, Pagano *et al.*, 2007)). Some studies have shown circulating endocannabinoid levels to be dependent on waist circumference or visceral adipose tissue to a greater extent than BMI. Given this, the second aim of this study was to examine whether there are relationships between FAAH and MGL activities in subcutaneous adipocytes and waist circumference. Finally, insulin sensitivity was measured to investigate whether, within a metabolically healthy sample, glycaemic regulation is related to FAAH and MGL activities in adipocytes.

4.3 Materials and Methods

4.3.1 Subjects

Ethical approval for this study was granted by the University of Nottingham Medical School Ethics Committee. Healthy volunteers from within the School of Graduate Entry Medicine and Health were recruited, via posters, and health screened as described in Section 2.3.1. After exclusion of 6 volunteers due to insufficient biopsy size or fasting blood glucose >5.6 mmol.L⁻¹, the data of 28 participants are reported in this study.

Volunteers were asked to make two visits. In the first, anthropometric measurements and blood pressure were taken. In the second, no more than one week later, fasting subcutaneous abdominal

adipose tissue and venous blood samples were taken as described in Sections 2.3.2, 2.3.3 and 2.3.4.

4.3.2 Homogenisation of adipocytes

Mature adipocytes were separated from the other adipose tissue cells using collagenase and centrifugation within one hour of collection (detailed in Section 2.2.1.). The adipocytes were then stored at -80°C until homogenisation as described in Section 2.2.2.

4.3.3 FAAH and MGL activity assay

The enzyme assays were performed as described in Section 2.5.

4.3.4 Protein assays

The protein concentration of the adipocyte fractions was measured using the Bradford method, as described in Section 2.5.3.

4.3.5 Blood serum assays

Fasting blood serum glucose, insulin, adiponectin, leptin and resistin concentrations were assayed for all volunteers, as described in Sections 2.7.1, 2.7.2, and 2.7.3 respectively. Glucose and insulin assays were performed within 6 months of sample collection. Adipokine assays were performed within 18 months of sample collection. The homeostatic model assessment (HOMA2-%S) figures were calculated using the HOMA2 model (www.dtu.ox.ac.uk).

4.3.5 Statistical analysis

GraphPad Prism Software was used to perform linear regression analyses and the Pearson correlation coefficient is reported.

4.4 Results

4.4.1 Subject characteristics

From an initial sample of 34 volunteers, 28 samples are reported on here. Of the excluded subjects, three had a fasting glucose $>5.6 \text{ mmol.L}^{-1}$ and three adipose samples were of insufficient size to obtain reproducible results. The physiological characteristics of this cohort are given in Table 4.1. The fasting serum glucose and insulin values indicate that these subjects were all metabolically healthy (American-Diabetes-Association, 1997; Alberti *et al.*, 2009), but the HOMA2-%S values indicate a range of insulin sensitivities and the BMI range is from lean ($<25.0 \text{ kg.m}^{-2}$) to obese ($>30.0 \text{ kg.m}^{-2}$). As would be expected, in this sample BMI correlated with the sum of skinfolds ($r=0.62$, $P<0.001$, Figure 4.1A), mean arterial blood pressure ($r=0.47$, $P<0.05$, Figure 4.1B), waist:hip ratio ($r=0.40$, $P<0.05$, Figure 4.1C) and waist circumference ($r=0.83$, $P<0.001$, Figure 4.1D), and had a non-significant negative trend with HOMA2-%S ($r=-0.33$, $P=0.09$, Figure 4.1E).

	Range	Mean \pm S.D.
Male:female ratio	1:1	
Age (years)	20-48	31 \pm 8.7
BMI (kg.m^{-2})	19.1-33.8	24.2 \pm 3.4
Systolic blood pressure (mmHg)	104-135	120.5 \pm 8.8
Diastolic blood pressure (mmHg)	60-91	73.4 \pm 7.2
MAP (mmHg)	77-106	89.1 \pm 6.2
Pulse (beat.min^{-1})	40-80	63.4 \pm 9.5
Waist circumference (cm)	69-114	82.2 \pm 10.7
Hip circumference (cm)	80-115	96.9 \pm 8.0
Waist:hip ratio	0.69-1.06	0.85 \pm 0.08
Sum of 7-point skinfolds (mm)	63-173	99.9 \pm 25.5
Tricep skinfold (mm)	7-32	15 \pm 6
Bicep skinfold (mm)	4-18	9 \pm 4
Abdominal skinfold (mm)	11-26	20 \pm 4
Suprailiac skinfold (mm)	5-25	17 \pm 6
Subscapular skinfold (mm)	7-36	16 \pm 6
Chest skinfold (mm)	3-18	9 \pm 4
Midaxillary skinfold (mm)	5-26	14 \pm 6
Serum glucose (mmol.L^{-1})	4.4-5.6	5.1 \pm 0.3
Serum insulin (mU.L^{-1})	1.8-13.1	5.7 \pm 3.2

HOMA2-%S	59.4-361.5	165.0±79.2
Serum adiponectin ($\mu\text{g}.\text{ml}^{-1}$)	3.55-21.28	10.93±5.03
Serum leptin ($\text{ng}.\text{ml}^{-1}$)	0.34-36.53	8.5±15.44
Serum resistin ($\text{ng}.\text{ml}^{-1}$)	5.38-32.07	11.49±7.19

Table 4.1 Subject characteristics

Physiological data of metabolically healthy human volunteers. Serum samples were taken from fasting subjects ($n=28$).

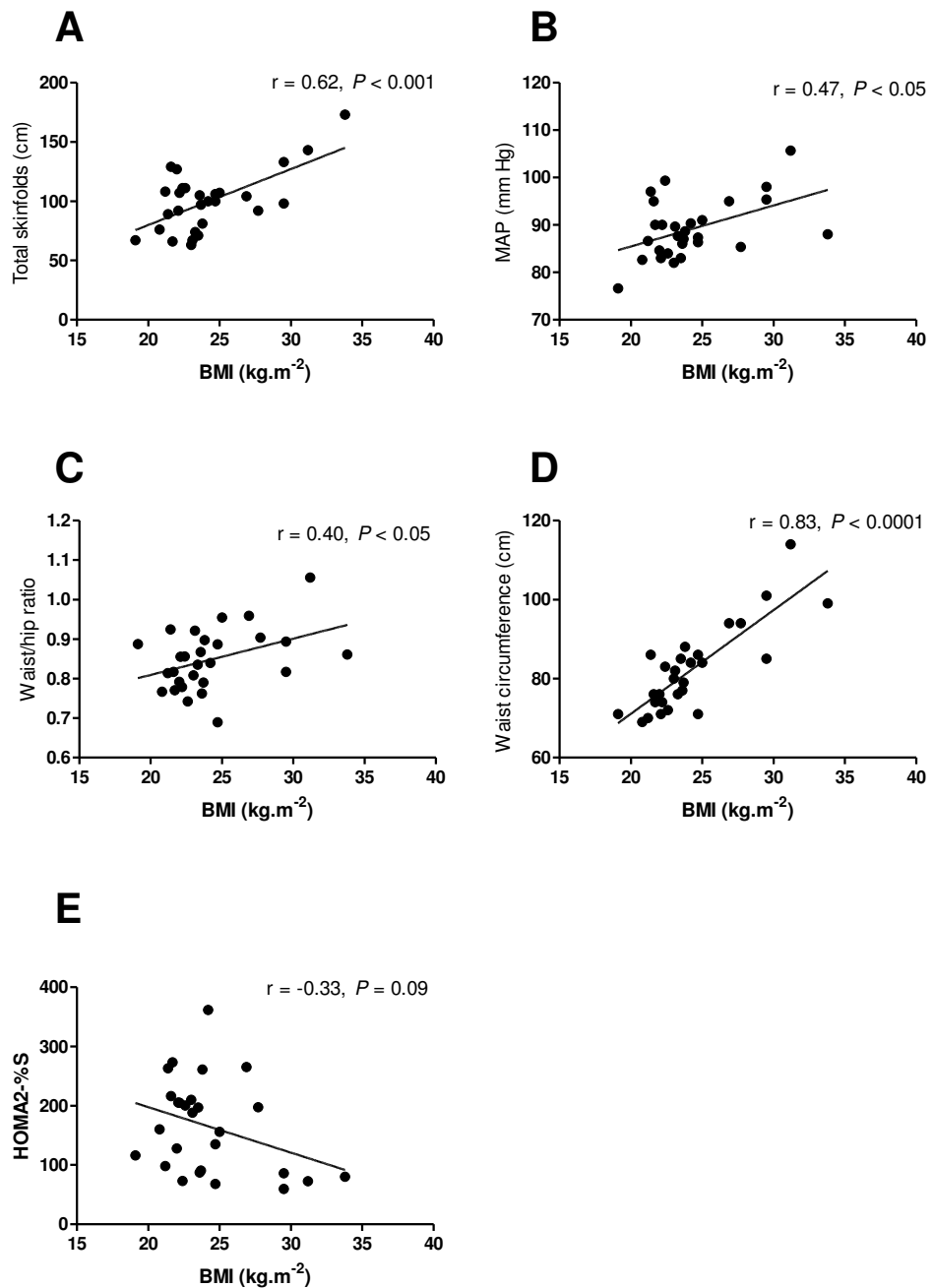


Figure 4.1 Correlations between BMI and other variables

The relationship between BMI and sum of skinfolds (A), mean arterial pressure (B), waist:hip ratio (C), waist circumference (D) and HOMA2-%S (E) in healthy humans. r and P values were obtained using correlation analysis.

4.4.2 Enzyme activity, BMI, body fat and circumferences

In these metabolically healthy subjects, FAAH activity in subcutaneous mature adipocytes correlated positively with BMI ($r=0.38$, $P<0.05$, Figure 4.2A) and with waist circumference ($r=0.43$, $P<0.05$, Figure 4.3A). FAAH activity did not correlate with calculated body fat percentage ($r=0.15$, $P=0.47$, Figure 4.2C). In contrast, MGL activity in subcutaneous mature adipocytes was not found to correlate with BMI ($r=-0.20$, $P=0.32$, Figure 4.2A) or waist circumference ($r=-0.08$, $P=0.71$, Figure 4.3B).

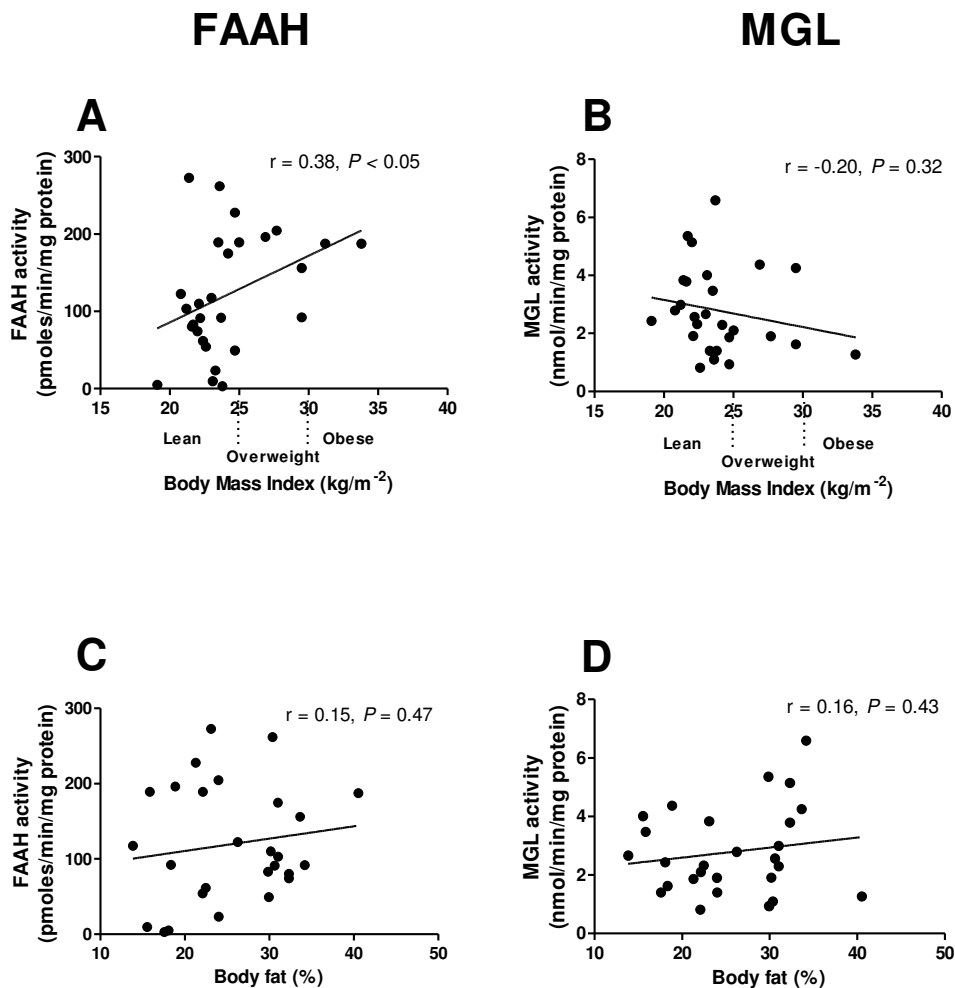


Figure 4.2 FAAH and MGL with body fat estimates

The relationship between FAAH and MGL activities in mature adipocytes isolated from abdominal subcutaneous adipose tissue and BMI (A,B) and body fat percentage (C,D). The Pearson correlation coefficient is reported..

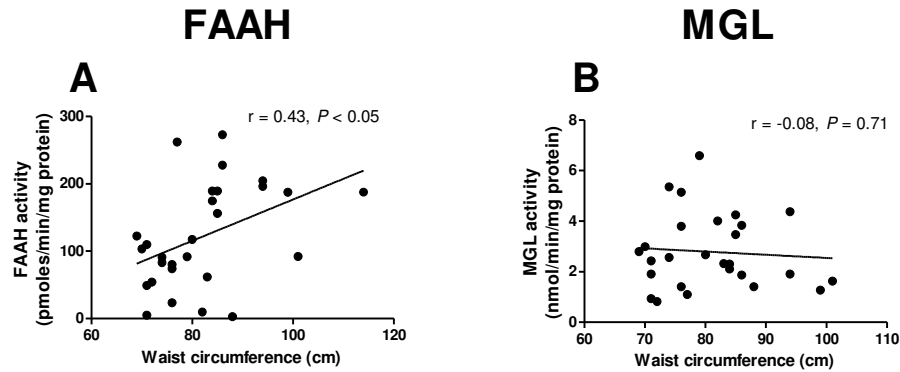


Figure 4.3 FAAH and MGL with waist circumference

The relationship between FAAH (A) or MGL (B) activities in mature adipocytes isolated from abdominal subcutaneous adipose tissue and waist circumference. The Pearson correlation coefficient is reported..

4.4.4 Enzyme activity and blood pressure

FAAH and MGL activities in abdominal subcutaneous adipocytes did not correlate with mean arterial pressure ($r=0.24, P=0.22$, Figure 4.4A; $r=0.17, P=0.39$, Figure 4.4B).

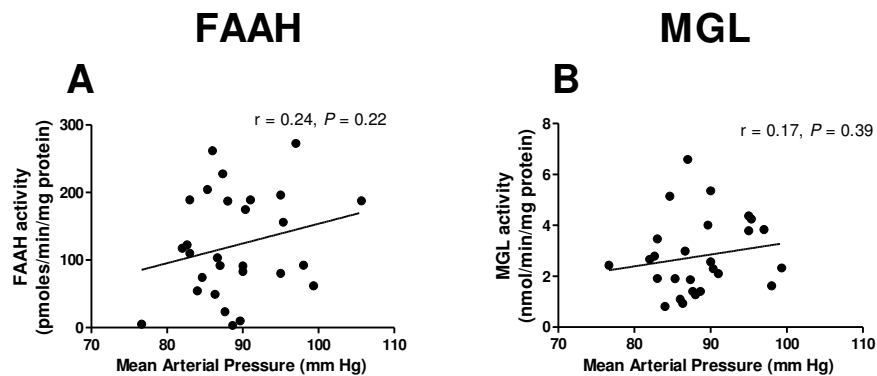


Figure 4.4 FAAH and MGL with blood pressure

The relationship between FAAH (A) or MGL (B) activities in mature human adipocytes isolated from abdominal subcutaneous adipose tissue and mean arterial blood pressure. The Pearson correlation coefficient is reported..

4.4.5 Enzyme activity and insulin sensitivity

HOMA2-%S did not show a relationship with FAAH or MGL activity in mature abdominal subcutaneous adipocytes ($r=0.06, P=0.78$, Figure 4.5A; $r=0.12, P=0.56$, Figure 4.5B).

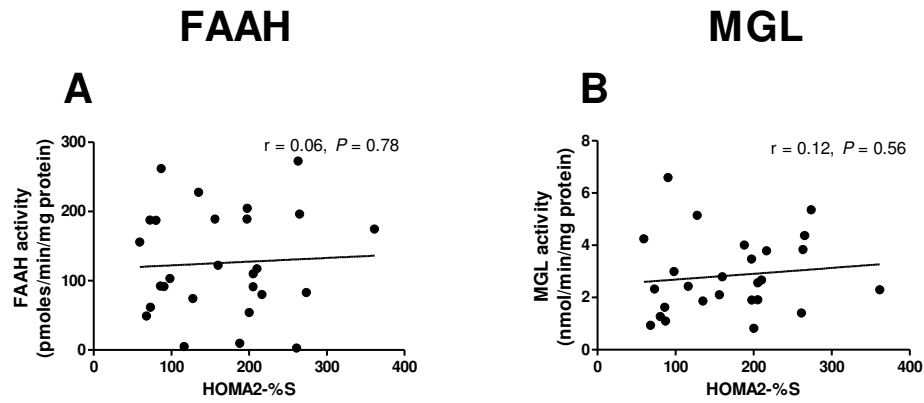


Figure 4.5 FAAH and MGL with insulin sensitivity

The relationship between FAAH (A) or MGL (B) activities in mature adipocytes isolated from abdominal subcutaneous adipose tissue and HOMA. The Pearson correlation coefficient is reported..

4.5 Discussion

There are several studies showing that the mRNA and protein levels of some components of the ECS are upregulated in subcutaneous adipose tissue in obesity. In addition to this, the results presented in Chapter 3 show that FAAH and MGL activities are increased in obese Zucker rats compared to lean rats. In light of this information, the principal aim of this study was to investigate whether the activities of FAAH and MGL, two key catabolic enzymes of the ECS, are altered with increasing BMI in otherwise healthy humans. The results show that FAAH activity in subcutaneous mature adipocytes increases with BMI and waist circumference. In contrast to this and the Zucker rat data, there is no relationship between MGL and BMI. Neither FAAH nor MGL activities correlate with insulin sensitivity in these healthy volunteers.

In several published studies, the mRNA levels of FAAH in adipose tissue have been compared between lean and obese humans, and major discrepancies have been reported over whether FAAH is upregulated (Murdolo *et al.*, 2007; Pagano *et al.*, 2007) or downregulated (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Lofgren *et al.*, 2007) in adipose tissue in obesity. In order to investigate this further in the current study FAAH activity, rather than mRNA, was measured, as mRNA levels do not always accurately reflect final protein levels (Bennetzen *et al.*, 2010) or enzyme activity. This is particularly important for FAAH, as post-transcriptional regulation of this enzyme has not been reported. This was done in subcutaneous mature adipocytes from subjects over a range of BMIs, from lean to obese. The results show that in adipocytes from metabolically healthy people FAAH activity increases with BMI. This is similar to the results seen in Zucker rats showing a correlation between FAAH activity and weight. This is in agreement with studies showing increased FAAH mRNA in the subcutaneous adipose of obese individuals compared to lean (Murdolo *et al.*, 2007; Pagano *et al.*, 2007), and also with a study in mice showing that diet-induced obesity led to increased expression of FAAH protein in subcutaneous adipose tissue (Starowicz *et al.*, 2008). However, other human studies have shown reported decreases in FAAH mRNA in obesity (Engeli *et al.*, 2005; Blüher *et al.*, 2006; Lofgren *et al.*, 2007). The reasons for conflicting results are currently unclear, but it should be noted that two of these studies used entirely female samples (Engeli *et al.*, 2005, Lofgren *et al.*, 2007), and the other reported on surgical patients (Blüher *et al.*, 2006). A further important distinction to draw is that in this study

isolated mature adipocytes were used rather than whole adipose tissue homogenates, and it has been shown that macrophages have significant FAAH expression (Sun *et al.*, 2005). In addition subjects representing a continuous range of BMIs were used in this study, as opposed to the discrete cohorts of lean and obese subjects used in many studies. This has allowed the inclusion of data from people with a BMI between 25 and 30 kg.m⁻², which is a group that has not been described previously.

The increase in adipocyte FAAH activity with increasing BMI may simply be part of a general upregulation of ECS tone in adipose tissue in obesity, as suggested by previous authors (Aurore and Gonthier, 2010; Bermudez-Silva *et al.*, 2010). If both the synthesis and degradation of anandamide are upregulated in balance, anandamide signalling and the functional effects of anandamide within the adipocyte are unlikely to be altered. This hypothesis is supported by a recent report that anandamide concentrations in subcutaneous adipose tissue do not differ between lean and metabolically healthy obese humans (Annuzzi *et al.*, 2010). Alternatively, FAAH may be upregulated in isolation. This would potentially reduce anandamide signalling both at CB₁ and CB₂ receptors, and at intracellular targets such as TRPV1 and PPARs. As missense mutations in the FAAH gene have been associated with an unfavourable metabolic profile in obese subjects (de Luis *et al.*, 2010b), the increase of FAAH activity with BMI reported here may be metabolically protective.

In some of the studies of the ECS in humans it has been found that adipose tissue distribution (subcutaneous versus visceral) is a better determinant of ECS expression than BMI or overall body fat percentage. For example, although circulating 2-AG levels have been reported to be increased in obesity, this has been shown to be accounted for largely by predominantly viscerally obese patients rather than subcutaneously obese (Blüher *et al.*, 2006). It is known that in humans of the same BMI, visceral adipose tissue accumulation confers greater metabolic and cardiovascular risk than excess subcutaneous adipose tissue (Despres *et al.*, 2008), but BMI does not distinguish between visceral and subcutaneous adipose tissue. For these reasons, in this study skinfold thicknesses and circumferences at various anatomical sites were measured to give an indication of fat distribution in the subjects. It was found that FAAH activity correlates with waist circumference. In general, this result is in keeping with studies showing that visceral obesity is a better predictor of circulating 2-AG levels than non-specific obesity (Engeli *et al.*, 2005; Blüher *et al.*,

2006) and may support the notion that central adipose accumulation is more significant in relation to the ECS than the amount of subcutaneous adipose tissue.

Humans with type 2 diabetes have increased plasma concentrations of anandamide and 2-AG compared to non-diabetic controls (Matias *et al.*, 2006). It has also been shown that FAAH mRNA is overexpressed in subcutaneous adipose tissue in obese humans and that similarly high expression was induced in healthy lean humans using the euglycaemic hyperinsulinaemic technique (Murdolo *et al.*, 2007). This is evidence that hyperinsulinaemia may directly cause FAAH upregulation. Considering this, in the present study fasting serum levels of insulin and glucose and HOMA2-%S (an estimation of insulin sensitivity) were analysed to establish whether there is any relationship with FAAH activity. In this sample of metabolically healthy humans, there was no correlation between FAAH activity in subcutaneous adipocytes and HOMA2-%S. The subjects in this study had fasting serum levels within healthy reference ranges, but a range of insulin sensitivities was observed. Despite using only humans with normal fasting blood glucose in this study, these findings are consistent with the results seen in the Zucker rats, in which FAAH activity did not correlate with blood glucose in rats ranging from healthy to fully insulin resistant. In order to further investigate whether there are relationships between insulin or glucose and FAAH activity in mature adipocytes, some patients with poor glycaemic regulation were included in the next study (see Chapter 5).

In mature adipocytes MGL has a primary role in lipid metabolism, specifically in the hydrolysis of monoglycerols to release glycerol and fatty acids (as described in Section 1.1.3.2). This explains the relatively high activity of MGL (~300 fold) compared to FAAH found in this study. The effects of MGL activity on 2-AG signalling are substantial, as demonstrated recently in mouse models, showing that both MGL gene deletion and systemic MGL inhibition lead to increased 2-AG levels in the brain and peripheral tissues, and desensitisation of brain CB₁ receptors (Chanda *et al.*, 2010; Schlosburg *et al.*, 2010). As MGL is not thought to be under hormonal control in triglyceride catabolism, it has not been extensively investigated in relation to obesity. However, MGL is highly important in 2-AG signalling in the ECS, and it has been shown that plasma 2-AG concentration in humans rises with obesity and in the Zucker rat study MGL activity in adipocytes increased with body weight. For these reasons,

MGL activity was analysed in this study with relation to BMI and other markers of adiposity. In contrast to the results seen with FAAH activity, MGL activity in subcutaneous adipocytes had no relationship with BMI, adiposity or glycaemic regulation. This is in agreement with at least one study in which the level of MGL mRNA in subcutaneous adipose tissue was not found to be different between lean and obese humans (Lofgren *et al.*, 2007) and another showing the same thing in visceral adipose tissue (Matias *et al.*, 2006). More generally, our findings are also in agreement with the observation that the rate of glycerol release from adipose tissue is the same in lean and obese subjects, in both fasting and fed states (Bolinder *et al.*, 2000). In contrast, another study has shown an increase in MGL mRNA in subcutaneous adipose tissue in obese humans compared to lean (Pagano *et al.*, 2007). It is also interesting that the results observed in humans in this study are different to the finding in the Zucker rats, in which MGL activity in subcutaneous adipocytes increased with body weight. Our findings in humans suggest that, with regard to adipocyte contribution to systemic 2-AG catabolism, the increase in circulating 2-AG observed in obese humans may be due to enhanced production rather than decreased degradation.

In summary, the results of this study show that FAAH activity in human subcutaneous mature adipocytes from healthy volunteers increases with BMI and waist circumference, but not with other markers of adiposity or metabolism. Conversely, MGL activity does not correlate with BMI or any other markers measured in this study. In the relatively metabolically healthy humans used in this study, neither FAAH nor MGL activities correlated with serum glycaemic markers, so in the following study obese patients with a range of metabolic dysfunction were investigated.

5. FAAH and MGL in mature adipocytes from obese patients

5.1 Introduction

Dysregulation of the peripheral ECS has been described in obesity, diabetes and other metabolic disorders in several published studies and in the work presented in this thesis. To summarise the results given so far, Chapter 3 showed that FAAH activity in adipocytes was increased in obesity, but not increased in the obese diabetic rats. This indicates that FAAH regulation in diabetes may differ from that in the obese but relatively insulin sensitive state. By contrast, MGL activity was increased in both obesity and in the obese diabetic rats compared to the lean rats. In Chapter 4 evidence was given that in healthy human subcutaneous adipocytes, FAAH activity increases with BMI, whereas MGL activity does not. However, the potential effects of metabolic disorder and diabetes were not investigated in this human sample as all subjects had normal fasting blood glucose concentrations.

The published studies that are relevant to this work have been described in detail in previous chapters. Very briefly, acute hyperinsulinaemia in lean humans has been found to increase FAAH mRNA expression in subcutaneous adipose tissue, but this acute response does not occur in obese humans with chronic hyperinsulinaemia and chronically elevated FAAH expression (Murdolo *et al.*, 2007). Furthermore, in humans with type 2 diabetes, plasma endocannabinoid levels are increased (Matias *et al.*, 2006), and endocannabinoid levels in subcutaneous adipose tissue are altered (Annuzzi *et al.*, 2010). These studies, along with the results of Chapter 3, suggest that insulin and/or diabetes affect FAAH, but to date, the effects of diabetes or metabolic syndrome on the activities of FAAH and MGL in human mature adipocytes have not been investigated.

The function and signalling of adipose tissue are affected by both total body mass and the site of adipose tissue deposition. This has been clearly demonstrated for hormones such as leptin and adiponectin (Sethi and Vidal-Puig, 2007), but in the case of the ECS the findings are not as well established. In normal weight humans CB₁ receptor mRNA has been reported to be higher in subcutaneous fat than visceral (Sarzani *et al.*, 2009; Bennetzen *et al.*, 2010), unchanged (Pagano *et al.*, 2007) or elevated in visceral compared to subcutaneous (Blüher *et al.*, 2006). However, in obese patients, CB₁ receptor expression may be higher in visceral fat than subcutaneous (Blüher *et al.*, 2006; Pagano *et al.*, 2007;

Sarzani *et al.*, 2009) or not different (Blüher *et al.*, 2006). As a further complicating factor, this may depend on whether the majority of excess adipose tissue is visceral or subcutaneous (Blüher *et al.*, 2006). FAAH mRNA has also been reported in visceral and subcutaneous adipose tissue in humans. One study has shown that FAAH mRNA levels do not differ between visceral and subcutaneous adipose tissue, even in obesity (Pagano *et al.*, 2007), but another found that FAAH mRNA is upregulated in visceral compared to subcutaneous depots (Blüher *et al.*, 2006). MGL mRNA was found to be elevated in subcutaneous compared to visceral adipose tissue, regardless of BMI (Pagano *et al.*, 2007). In the published literature to date, there are few studies in which FAAH and MGL mRNA have been measured in different adipose tissue depots, and none in which the activities of the enzymes have been measured in mature adipocytes.

5.2 Aims

This study was based on the findings of Chapters 3 and 4, which showed that FAAH and MGL activities differed between the lean, obese and obese diabetic rats, and that FAAH activity in human subcutaneous adipocytes is increased with BMI. This study was also designed to address the contradictions in published studies which show various effects of diabetes or insulin on the ECS in adipose tissue. Therefore, clinically obese patients with varying degrees of metabolic dysfunction were recruited. The first aim of the study was to investigate whether FAAH or MGL activities in isolated mature adipocytes correlate with BMI, waist circumference or long-term glycaemia. Another closely associated aim, using the same data set, was to determine whether FAAH or MGL activities are different between relatively healthy obese patients, and those with metabolic syndrome or diagnosed type 2 diabetes. Finally, as Chapter 3 showed no difference in enzyme activities in adipocytes from different adipose tissue depots, the activities of FAAH and MGL between paired subcutaneous and visceral mature adipocytes were compared.

5.3 Materials and Methods

5.3.1 Patient recruitment

The study was approved by Derbyshire Regional Ethics Committee and Royal Derby Hospital Trust, and written informed consent was obtained from all patients. Patients were recruited to this study prior to surgery as described in Section 2.4.1. Anthropometric measurements were taken as in

Section 2.4.2. Briefly, the sample comprised patients undergoing laparoscopic bariatric surgery for weight loss, and a few patients undergoing laparoscopic cholecystectomies. This latter group of patients was recruited in order to include data on people with BMI $<40 \text{ kg.m}^{-2}$. Patients were selected to represent three subgroups of the obese population, which have been extensively characterised in previous studies. These are obese patients who are metabolically healthy; obese patients with metabolic syndrome; and obese patients with type 2 diabetes.

5.3.2 Healthy, metabolic syndrome and diabetic grouping

The patients in this study were assigned to one of three groups after recruitment and sample collection: metabolically healthy, metabolic syndrome and diabetic. Patients were included in the type 2 diabetes group according to clinical diagnosis prior to surgery. The remaining patients were then assigned to the metabolic syndrome group or healthy group according to criteria published jointly by several major associations and groups (Alberti *et al.*, 2009). Metabolic syndrome is diagnosed if any 3 of the 5 major factors associated with increased metabolic and cardiovascular risk are present. These factors are: waist circumference $\geq 94 \text{ cm}$ (male) or $\geq 80 \text{ cm}$ (female); blood serum triglyceride $\geq 1.7 \text{ mmol.L}^{-1}$; blood serum HDL-cholesterol $<1 \text{ mmol.L}^{-1}$ (male) or $<1.3 \text{ mmol.L}^{-1}$ (female); systolic blood pressure $\geq 130 \text{ mm Hg}$ and/or diastolic blood pressure $\geq 85 \text{ mm Hg}$; and fasting blood serum glucose $\geq 5.6 \text{ mmol.L}^{-1}$. The prescription of drugs to control dyslipidaemia (fibrates or nicotinic acid), blood pressure or hyperglycaemia negates the requirement for the relevant factor to be outside these ranges. All patients in this study had a waist circumference $>100 \text{ cm}$, but patients with only one other marker of metabolic syndrome were assigned to the obese but metabolically healthy group. It should be noted that these patients cannot be considered a healthy sample of the general population, but they do represent a relatively healthy sample of the obese population.

5.3.3 Medications

At the time of surgery 14 of the patients were taking prescription drugs to treat dyslipidaemia, hypertension and/or diabetes (see Table 5.1). In the relatively healthy group, one patient was taking a statin, one patient an angiotensin converting enzyme (ACE) inhibitor, and two patients were taking both ACE inhibitors and thiazide diuretics. In the metabolic

syndrome group one patient was taking a statin, one patient an ACE inhibitor, one patient an ACE inhibitor plus a β_1 adrenoceptor antagonist and one patient an ACE inhibitor plus a thiazide diuretic. In the diabetic group five patients were prescribed metformin. Of these, one was also taking an ACE inhibitor, one was taking a statin and one was taking an ACE inhibitor plus a statin. One patient in the diabetic group was prescribed a thiazide diuretic only.

5.3.4 Adipose tissue sample collection

Subcutaneous and omental adipose tissue biopsies were obtained from patients during surgery as described in Section 2.4.3.

5.3.5 Adipocyte isolation and enzyme assays

The adipose tissue samples were collected and stored as described in Section 2.4.3. The samples were thawed at a later date and the mature adipocytes isolated and homogenised as in Sections 2.2.1 and 2.2.2. FAAH and MGL assay were performed on the adipocyte homogenate fractions as described in Section 2.5.

5.4 Results

5.4.1 Patient characteristics

Subcutaneous adipose tissue samples were obtained from 27 patients, and visceral adipose tissue samples were also obtained from 14 of these patients. The physiological characteristics of these patients are given in Table 5.1, which shows the sample divided into three groups. These groups are: patients with clinically diagnosed type 2 diabetes ($n=10$), patients without diabetes but with at least three markers of metabolic syndrome (Alberti *et al.*, 2009) ($n=11$), and patients without diabetes and with only one or two markers of metabolic syndrome ($n=6$). Between these groups, age, BMI, fasting serum insulin concentration, HOMA and mean arterial pressure did not differ. The mean fasting serum glucose concentration and HbA1c were higher in the diabetic group than both the healthy and metabolic syndrome groups ($P<0.05$). Patients in all groups were prescribed similar medications for dyslipidaemia and hypertension, but 5 patients in the diabetic group were taking hypoglycaemic medication compared to none in the healthy and metabolic syndrome groups (see Table 5.1).

	Metabolically healthy (n = 6)	Metabolic syndrome (n = 11)	Diagnosed diabetes (n = 10)
Age (years)	44.0±4.7	44.5±3.3	45.5±2.7
BMI (kg.m⁻²)	44.0±4.7	46.3±1.7	44.8±1.5
Insulin (mU.L⁻¹)	12.2±3.1	14.0±2.3	15.9±1.9
Glucose (mmol.L⁻¹)	5.0±0.2~	5.1±0.2*	8.7±1.4~*
HbA1c (%)	5.8±0.1 [†]	5.8±0.3 [#]	7.9±0.7 ^{†#}
HOMA2-%S	85.8±26.2	71.4±14.2	50.4±6.8
MAP (mmHg)	96.1±4.8	96.3±4.3	104.8±3.6
Dyslipidaemia medication	1 (statin)	1 (statin)	2 (statin)
Hypertension medication	0	0	5 (metformin)
Hypertension medication	3 (1: ACE inhibitor; 2: ACE inhibitor + thiazide diuretic)	3 (1: ACE inhibitor; 1: ACE inhibitor + β_1 antagonist; 1: ACE inhibitor + thiazide diuretic)	3 (2: ACE inhibitor; 1: thiazide diuretic)

Table 5.1 Characteristics of patients

Physiological characteristics of the subjects included in this study. Values given as mean±S.E.M. Values marked with the same characters are significantly different from each other. Data analysed using ANOVA and Bonferroni's multiple comparison test, $P < 0.05$ for all significant values.

5.4.2 Enzyme activities, BMI and waist circumference

In this sample of obese patients, neither FAAH activity (Figure 5.1A) nor MGL activity (Figure 5.1B) in subcutaneous adipocytes correlated with BMI. Similarly waist circumference did not correlate with FAAH (Figure 5.1C) or MGL (Figure 5.1D) activity.

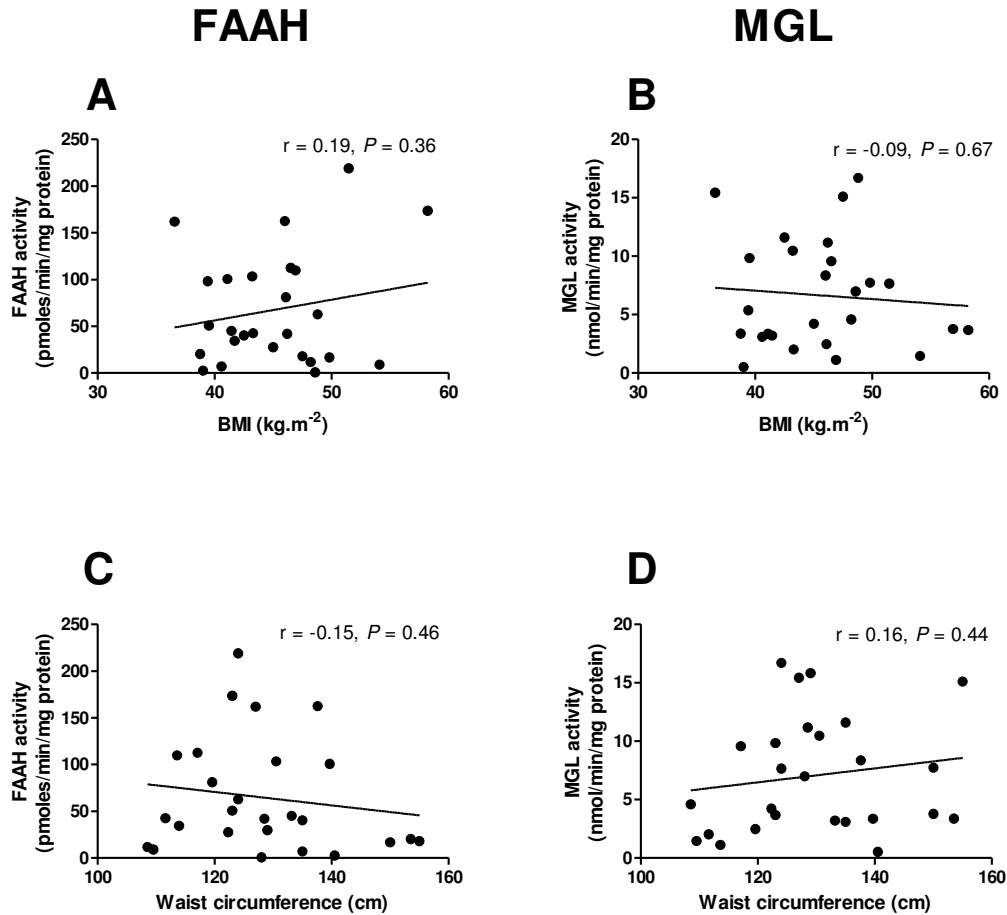


Figure 5.1 FAAH and MGL with BMI and waist circumference

The relationship between FAAH or MGL activities in subcutaneous adipocytes from obese humans and BMI (A,B) and waist circumference (C,D). The Pearson correlation coefficient is reported.

5.4.3 Enzyme activities and HbA1c

There were no correlations between FAAH or MGL activities and HbA1c (Figure 5.2).

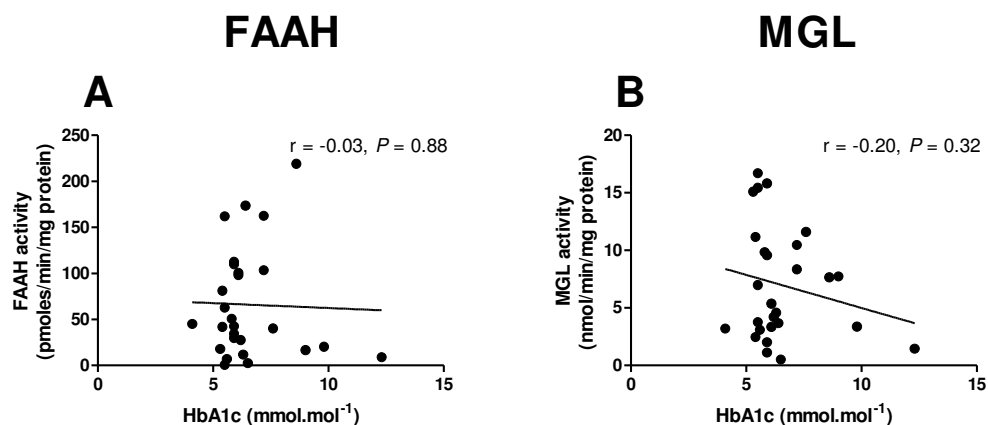


Figure 5.2 FAAH and MGL with HbA1c

The relationship between FAAH (A) or MGL (B) activities in subcutaneous adipocytes from obese humans and HbA1c. The Pearson correlation coefficient is reported.

5.4.4 Enzyme activities in different metabolic groups

The patients in this study were divided into healthy, metabolic syndrome and diabetic groups. FAAH activity in subcutaneous adipocytes did not differ between the three groups (Figure 5.3A). Similarly, MGL activity was not different between the groups (Figure 5.3B).

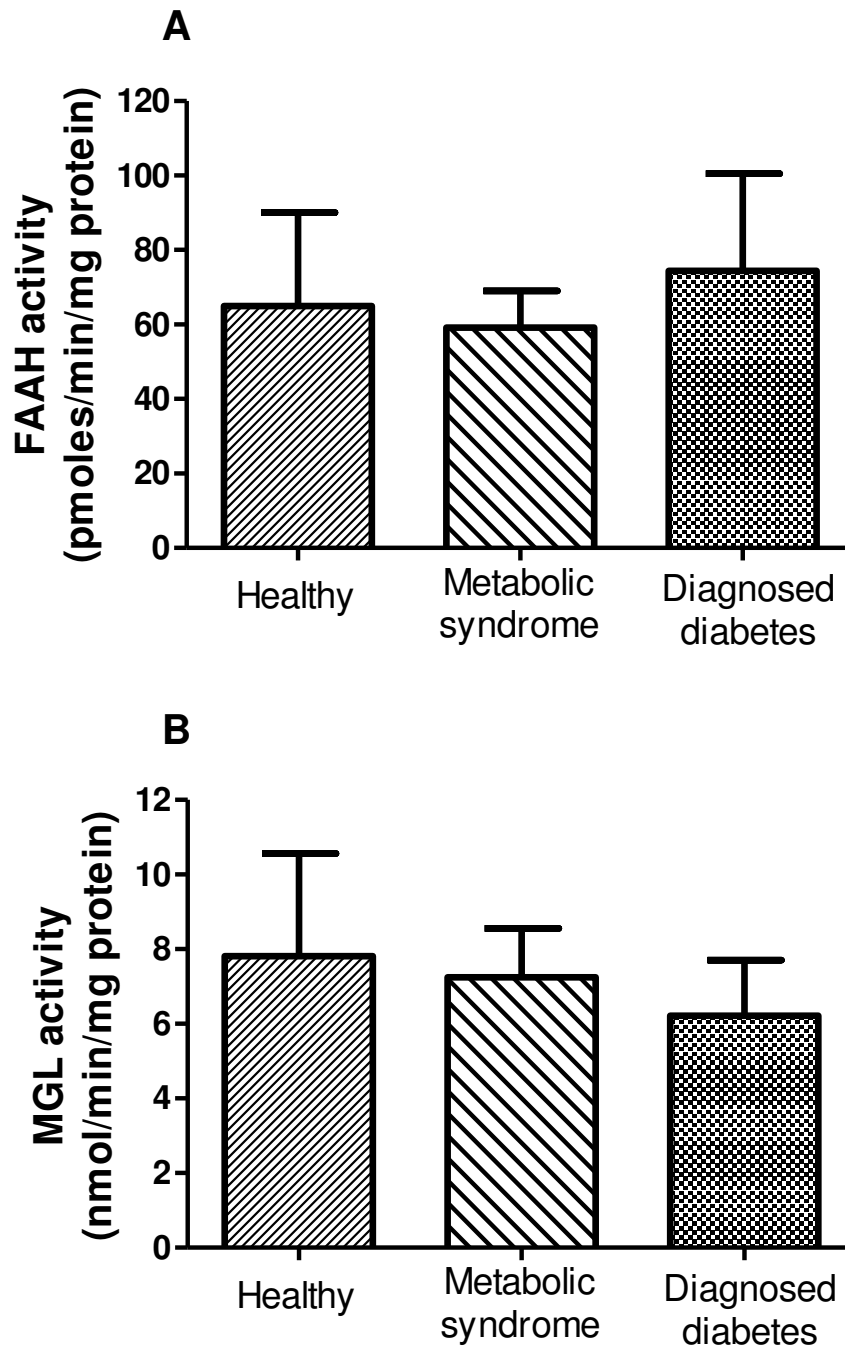


Figure 5.3 FAAH and MGL in three metabolic groups

FAAH (A) and MGL (B) activities in subcutaneous adipocytes from obese humans. Subjects were assigned to one of three groups based on the following criteria: healthy <2 components of metabolic syndrome ($n=6$); metabolic syndrome ≥ 3 components of metabolic syndrome ($n=11$); diagnosed type 2 diabetes with or without metabolic syndrome ($n=10$). Data are presented as means, with error bars representing S.E.M., and were analysed using one way ANOVA and Bonferroni's multiple comparison test.

5.4.5 Enzymes in subcutaneous and visceral adipocytes

In this human study, FAAH activity did not differ between subcutaneous and visceral adipocytes (69.3 ± 14.5 vs 70.3 ± 25.1 pmoles.min⁻¹.mg protein⁻¹, $n=14$, Figure 5.4A). The results were the same for MGL, with no difference between subcutaneous and visceral adipocytes (9.0 ± 2.3 vs 12.7 ± 1.9 nmoles min⁻¹.mg protein⁻¹, Figure 5.4B). Correlation studies showed that there was no relationship between FAAH activity in subcutaneous adipocytes and FAAH activity in visceral adipocytes (Figure 5.4C). Similarly, there was no correlation between MGL activities in adipocytes from these two adipose tissue depots (Figure 5.4D).

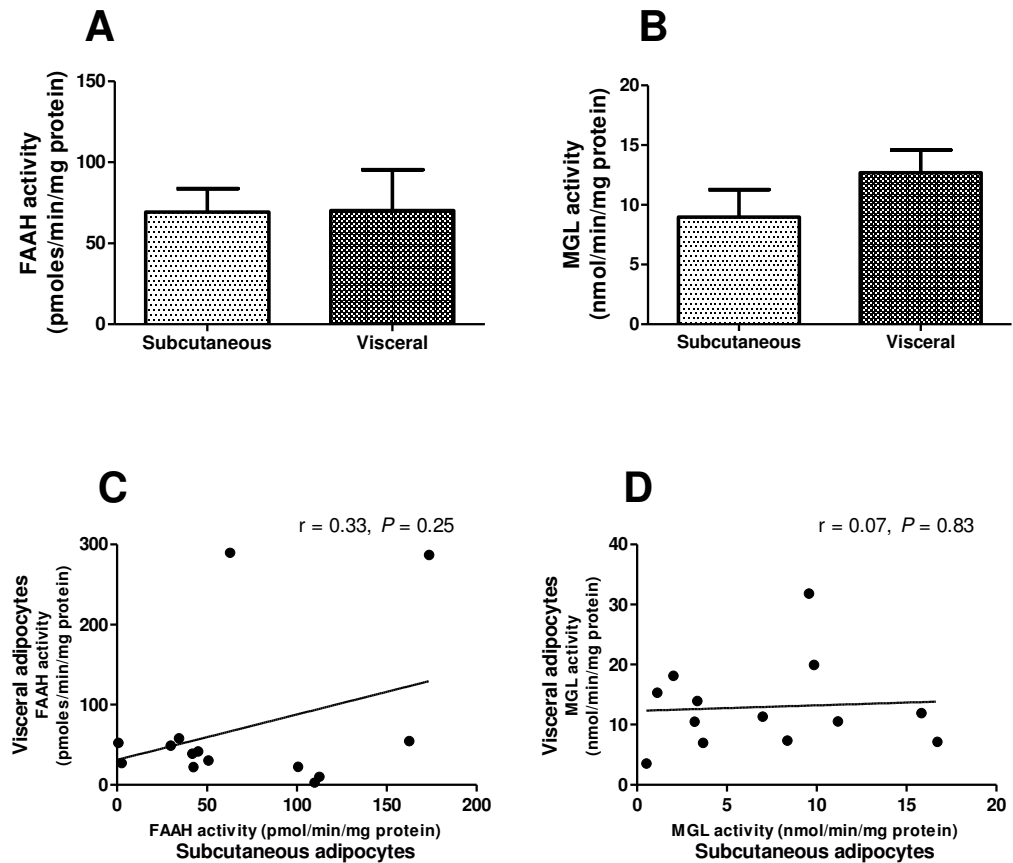


Figure 5.4 FAAH and MGL in subcutaneous and visceral adipocytes

FAAH (A) and MGL (B) activities in paired samples of subcutaneous and visceral mature adipocytes from obese humans ($n=14$). Data are presented as means, with error bars representing S.E.M., and were analysed using Student's paired t test. Relationships between FAAH activity in subcutaneous and visceral adipocytes (C) and MGL activity in subcutaneous and visceral adipocytes (D). The Pearson correlation coefficient is reported.

5.5 Discussion

The results of the previous chapters indicate that FAAH and MGL activities in isolated mature adipocytes are altered in obesity and diabetes in Zucker rats, and FAAH is upregulated in subcutaneous adipocytes in obese but otherwise healthy humans. In light of these findings, the primary aim of the study presented here was to determine whether FAAH or MGL activities in subcutaneous adipocytes are altered in diabetes or other metabolic dysfunction in clinically obese humans. Given that FAAH activity correlated with BMI and waist circumference in healthy humans, relationships between the enzyme activities and these anthropometric measurements were also investigated. In the obese Zucker rats, FAAH and MGL activities were not different between subcutaneous and visceral adipocytes, despite the differing metabolic roles of these adipose tissue depots. Therefore, the second aim of this study was to determine whether these enzyme activities differed between sites in obese humans. The main finding of this study was that FAAH and MGL activities were not altered in patients with metabolic syndrome or diabetes compared to obese but relatively healthy patients. Finally, FAAH and MGL activities did not differ between paired subcutaneous and visceral (omental) adipocytes.

The results presented in Chapter 4 showed that FAAH activity increased not only with BMI, but with waist circumference. In another study, obese humans with predominantly subcutaneous adipose tissue were shown to have higher FAAH mRNA in adipose tissue than viscerally obese patients of the same BMI (Blüher *et al.*, 2006). For these reasons, BMI and adipose tissue distribution were included in the analysis in the obese patients used in this study. The results show that FAAH activity in subcutaneous adipocytes from obese humans does not correlate with BMI or waist circumference. The patients used in this study were all clinically obese, but had a range of BMIs between 36.6 and 58.2 kg.m⁻². Given that in Chapter 4 it was found that FAAH activity in mature adipocytes from subcutaneous adipose tissue correlates with BMI, it might be expected that this relationship would be seen in obese patients, and it is interesting to note that this is not the case. In a study in which FAAH mRNA was found to be downregulated in subcutaneous adipose tissue in obese patients compared to lean, a 5% reduction in total body weight following calorie restriction did not affect the mRNA levels of FAAH (Engeli *et al.*, 2005). A similar finding was reported for anandamide concentration and FAAH mRNA in subcutaneous adipose tissue, as weight loss in obese humans did

not alter their expression levels (Bennetzen *et al.*, 2011). Overall, this indicates that in the obese population, weight loss must be larger before alterations in the ECS are seen and there may not be significant differences in the expression of the ECS between obese patients. A possible conclusion from these findings is that in human obesity FAAH activity in adipocytes is dysregulated in a way that is not proportional to the level or distribution of adiposity. It would have been interesting to include the results from Chapter 4 in the correlation studies to investigate this further, but this was not appropriate as the samples were obtained very differently between the two studies.

Blood plasma 2-AG levels are increased in obesity, but to date MGL activity in human adipocytes from obese humans has not been reported. More specifically it has been shown that circulating 2-AG levels are higher in viscerally obese humans than in subcutaneously obese subjects (Blüher *et al.*, 2006). Therefore in this study MGL activity in adipocytes was analysed in relation to BMI and waist circumference. The results for MGL in this study are the same as for FAAH, in that MGL activity in the subcutaneous mature adipocytes does not correlate with BMI or waist circumference. These findings are in agreement with the results seen in the Zucker rats, as there was no difference in MGL activity between the two strains of obese Zucker rat, which between them covered a continuous range of obese body mass. In another study, MGL mRNA in subcutaneous adipose tissue did not differ after weight loss in obese humans (Bennetzen *et al.*, 2011). Together with the results of Chapter 4, these findings indicate that MGL in human adipocytes does not vary with BMI or adipose tissue distribution. Furthermore, these results raise the possibility that differences in circulating and adipose tissue levels of 2-AG in obese humans (and subsets of subcutaneously and viscerally obese humans) may be due to alterations in the synthesis of 2-AG rather than degradation. This is speculative however, as the contribution that MGL activity in mature adipocytes makes to the reduction of circulating 2-AG has not been reported.

In Chapter 3 a wide range of blood glucose concentrations were seen, but glucose did not correlate with FAAH activity. Likewise, in Chapter 4 insulin sensitivity did not have a relationship with FAAH activity. Several authors have suggested that insulin has a role in peripheral endocannabinoid regulation (Murdolo *et al.*, 2007; Di Marzo *et al.*, 2009), but this has not yet been investigated with regard to FAAH activity. To

address this issue, in the current study obese patients with a range of fasting glucose and insulin concentrations were recruited. The results show that FAAH activity in mature adipocytes does not correlate with HbA1c. This finding is in keeping with the results of the Zucker rats and healthy humans, showing no relationship between glucose or insulin and FAAH activity. In the current study, HbA1c was measured, which is a marker of long-term (approximately 3 month) glycaemic regulation, whereas the random blood glucose concentration reported in the Zucker rat study is a better indicator of short term glucose handling. The HOMA values in this sample of patients indicate that, although all of the patients were clinically obese, they ranged from insulin sensitive to insulin resistant. In another study, FAAH mRNA levels in subcutaneous adipose tissue from lean and obese humans with a range of insulin sensitivities were measured. FAAH mRNA was found to correlate negatively with fasting plasma glucose, fasting insulin and glucose infusion rate during a euglycaemic-hyperinsulinaemic clamp in men but not in females (Blüher *et al.*, 2006). This may be an important finding given that the majority of the sample used in the current study was female.

There are several reports that the ECS is dysregulated in type 2 diabetes. For example, anandamide levels are increased in both blood (Matias *et al.*, 2006) and subcutaneous adipose tissue (Annuzzi *et al.*, 2010) in obese diabetic humans compared to obese metabolically healthy humans. This relationship was investigated in the Zucker rats and it was found that FAAH activity in the subcutaneous adipocytes did not differ between the obese and obese diabetic rats. This was not explored further in Chapter 4 as all of the humans used were metabolically healthy. The patients in this present study were assigned to healthy, metabolic syndrome and type 2 diabetic groups according to clinical diagnosis of type 2 diabetes and recent criteria for metabolic syndrome (Alberti *et al.*, 2009). The results presented here show that there is no difference in FAAH activity in the subcutaneous mature adipocytes between the healthy, metabolic syndrome and type 2 diabetic patients in this sample. This finding is the same as that observed in the Zucker rats, in which FAAH activity in the subcutaneous adipocytes did not differ between the obese and obese diabetic rats. These results suggest that the reported increases in anandamide levels in adipose tissue in diabetes (Annuzzi *et al.*, 2010) are not due to a decreased rate of anandamide degradation. It should be noted that in practice there is not clear separation between the three

metabolic groups defined in the results presented here. Many of the diabetic patients also fulfil the criteria for metabolic syndrome (Després *et al.*, 2008; Alberti *et al.*, 2009) and the results from this sample of patients are complicated by the range of diseases and medications included. Also, the diabetic patients, due to tight adherence to medical advice and medication, achieved better glycaemic control in the period preceding surgery than some patients who do not have overt diabetes or receive glycaemic therapy. In addition, due to pharmacological interventions a very low number of patients in this study had a high fasting insulin concentration. By contrast, in the study showing adipose tissue anandamide to be upregulated in obese diabetic humans, the patients were not taking any glycaemic or hypolipidaemic drugs (Annuzzi *et al.*, 2010). These findings suggest that any influence of diabetes or insulin sensitivity may only be observed in uncontrolled diabetes. In itself, this is interesting and may support the evidence for a relationship between the ECS and insulin sensitivity which has been described previously.

As has been reported for anandamide, circulating 2-AG concentrations have been found to be increased in obese diabetic humans compared to obese healthy humans (Matias *et al.*, 2006). However, in subcutaneous adipose tissue 2-AG concentrations have been reported to be decreased in diabetes (Annuzzi *et al.*, 2010). In the Zucker rats, MGL activity in mature subcutaneous adipocytes did not differ between the obese and obese diabetic rats, but in light of the studies showing altered 2-AG concentrations in human diabetes, MGL activity was measured in the current study. The results presented in this chapter are in agreement with the Zucker rat study, in that MGL activity in subcutaneous adipocytes does not differ between the relatively metabolically healthy, metabolic syndrome and diabetic patients. As with FAAH, these novel results suggest that altered 2-AG concentrations in adipose tissue in diabetes (Annuzzi *et al.*, 2010) are not due to increased 2-AG degradation by mature adipocytes.

In terms of lipid metabolism and cardiovascular risk, subcutaneous and visceral adipose tissue are often considered to be different, with visceral adipose tissue generally having greater metabolic activity and a greater impact on cardiovascular health (Wajchenberg, 2000). Differences in adipose tissue between different depots have also been reported in various components of the ECS. For example, in obese humans CB₁ receptor mRNA has been reported to be higher in visceral adipose tissue than subcutaneous (Pagano *et al.*, 2007; Sarzani *et al.*, 2009). No studies

have been published on the activities of FAAH or MGL in adipocytes from different adipose tissue depots, so this was included in the current study. The results of this chapter show that in paired subcutaneous and visceral adipocytes, neither FAAH nor MGL activity is affected by adipose tissue depot in obese humans. Additionally, there is no correlation between the enzyme activities in subcutaneous and visceral adipocytes. These results match those observed in the obese and obese diabetic Zucker rats (see Chapter 3). With regard to human studies, similar results have been reported in that FAAH mRNA levels were not found to be different in subcutaneous and visceral adipose tissue in obese humans (Pagano *et al.*, 2007). However, another study has shown that FAAH mRNA levels are higher in visceral than subcutaneous adipose tissue (Blüher *et al.*, 2006). Only one published study has reported MGL mRNA expression, and the result of this was that MGL is downregulated in visceral adipose tissue (Pagano *et al.*, 2007). The results of this chapter indicate that the rate of endocannabinoid degradation does not differ between visceral and subcutaneous mature adipocytes. It may be that differences in the stromal-vascular fraction between depots may account for the overall changes in mRNA observed in other studies, as in the current only mature adipocytes were used. It is interesting that in this study neither FAAH nor MGL activity correlated between adipocytes from the two adipose tissue depots tested. In another study, FAAH mRNA in adipose tissue was found to correlate with a high level of significance between visceral and subcutaneous depots. However, in that study lean and obese patients were included and whole adipose tissue was used (Blüher *et al.*, 2006). The results presented here indicate that in obese humans the rate of endocannabinoid degradation in adipocytes does not tend to be higher in visceral adipocytes than subcutaneous adipocytes.

In summary, several previous studies have shown that in obese humans circulating endocannabinoid levels and components of the ECS in adipose tissue are altered by insulin or diabetes. However, there are contradictions in the literature to date, and the effects of metabolic function on FAAH and MGL activities in mature adipocytes have not been reported. The results presented in this chapter show that FAAH and MGL activities in subcutaneous adipocytes from clinically obese humans are not altered in relation to BMI, adipose tissue distribution or insulin sensitivity. It has also been shown that these enzymes are not directly affected by metabolic syndrome or diabetes. Furthermore, no differences in activity

were identified between subcutaneous and visceral adipocytes. These results indicate that any alterations in endocannabinoid signalling in metabolic disorders, including type 2 diabetes, are not regulated by FAAH or MGL in mature adipocytes.

6. The effects of endocannabinoids on adipokine secretion in cultured human adipocytes

6.1 Introduction

The culture of human adipocytes has been improved considerably in recent years (Matsumoto *et al.*, 2008). Human adipocytes in culture are morphologically distinct from those taken from biopsies, but according to one study their function is comparable (Nobusue *et al.*, 2008). Cultured preadipocytes which are induced to differentiate become rounded and filled with many small lipid droplets. By contrast, mature adipocytes are spherical and almost completely filled with one large lipid droplet (Jiang *et al.*, 2007). If mature adipocytes are cultured, the volume of intracellular lipid decreases and after 12 days in culture their morphology closely resembles that of cell culture differentiated preadipocytes (Van *et al.*, 1976). In many studies, the 3T3-L1 murine fibroblast cell line is used. The advantages of these cells are that they proliferate at a significantly faster rate than human adipocytes and differentiation into mature adipocytes takes approximately a week less (Fowler *et al.*, 2009). However, few data are available on the comparability of this cell line to human adipocytes, particularly with regard to ECS expression and regulation.

To date relatively few papers regarding the effects of the ECS in cultured human adipocytes have been published, although it is clear that there are interactions between insulin stimulation and endocannabinoid turnover. There is some evidence that cannabinoid receptor activation in adipocytes increases insulin-induced glucose uptake. This has been demonstrated in differentiated murine 3T3-L1 cells in culture with both anandamide (Gasperi *et al.*, 2007) and THC (Gallant *et al.*, 2009). This has also been reported in human adipocytes in culture, using WIN-55,212 (Pagano *et al.*, 2007). The strength of activation with THC (10µg/ml) is such that glucose uptake occurs even in insulin resistant cells (Gallant *et al.*, 2009). The use of rimonabant has shown that insulin-stimulated glucose uptake is not directly CB₁ mediated (Pagano *et al.*, 2007).

It has also been demonstrated that insulin affects the ECS in adipocytes. In differentiated 3T3-L1 cells, an 8-hour exposure to high concentrations of insulin was found to increase FAAH expression, but MGL expression was significantly elevated only after 24 hours' treatment. As might be expected, in insulin resistant cells, insulin failed to raise FAAH mRNA. By contrast, MGL expression remained chronically raised once

insulin resistance had been induced and it was not further increased by acute insulin treatment. A 24-hour treatment with insulin reduced intracellular concentrations of anandamide and 2-AG by around a third (D'eon *et al.*, 2008). These results were supported by similar findings using DIO mice (D'eon *et al.*, 2008).

Interactions between adipokines and the ECS in adipocytes have also been reported. The consequences of these interactions could be important, given that two of the adipokines in particular, adiponectin and leptin, are known to have significant roles in whole body metabolic homeostasis. In one study using 3T3-L1 cells, production of anandamide and 2-AG was suppressed by both acute and chronic treatment with leptin (Matias *et al.*, 2006). Another study involving leptin showed that human subcutaneous adipocytes in culture produce PEA in concentrations at least 10-fold higher than anandamide or 2-AG. Leptin treatment of the cells for 1 hour was found to decrease this expression by 27%. In contrast, PEA treatment of the adipocytes did not significantly affect leptin or adiponectin concentrations (Gonthier *et al.*, 2007). Similarly, in cultured human adipocytes neither the cannabinoid receptor agonist WIN-55,212 nor rimonabant were found to affect leptin or adiponectin mRNA expression, but adipokine secretion was not measured (Pagano *et al.*, 2007). In contrast, CB₁ receptor stimulation using HU-210 was shown to reduce adiponectin mRNA expression in differentiated 3T3-L1 cells, and rimonabant markedly increased adiponectin mRNA in these cells (Matias *et al.*, 2006).

It has been reported that adipose tissue explants in culture secrete resistin (Fain *et al.*, 2003), but the mechanism of resistin secretion from adipose tissue remains debated. Mature adipocytes have been reported to secrete resistin in culture up to 48 hours after biopsy (McTernan *et al.*, 2003), but this has not been demonstrated in other studies or in adipocytes cultured for longer time periods. Some studies have also reported resistin mRNA in preadipocytes (Kim *et al.*, 2001; Janke *et al.*, 2002), but secretion of the protein has not been reported. Other studies have concluded that the majority of secreted resistin is produced by non-adipocyte cells (Fain *et al.*, 2003; Kaser *et al.*, 2003). Interactions between the ECS and resistin secretion have not been reported, and as yet no studies have investigated whether anandamide and 2-AG, known to be increased in obesity, alter adiponectin or leptin secretion.

6.2 Aims

The aim of this study was to investigate whether endocannabinoids (anandamide and 2-AG) affect the secretion of adipokines from human adipocytes in culture under normal physiological conditions and under high glucose and/or insulin conditions. These conditions were chosen to model hyperglycaemia and/or hyperinsulinaemia. The adipokines tested were adiponectin and leptin, as these are known to have roles in metabolism and their dysregulation in obesity has been characterised. Resistin was also included, as this protein may have a role in inflammatory processes and possibly in metabolic signalling, but its secretion from human adipocytes and preadipocytes remains debated.

6.3 Materials and Methods

6.3.1 Adipocyte culture

Human abdominal subcutaneous preadipocytes pooled from women undergoing cosmetic liposuction were purchased at passage 2 (Promocell, UK). These were cultured and differentiated in Promocell adipocyte media as described in Section 2.8.1. Briefly, the preadipocytes were grown in fibronectin coated 24-well plates until confluent, then induced to differentiate for 3 days. The adipocytes were left to differentiate for 12-14 days, with media changes every 2-3 days, before the treatments were initiated. For the resistin experiments, preadipocytes were also used. In this case the preadipocytes were treated as soon as the plates were confluent.

6.3.2 Insulin and glucose

The differentiated adipocytes (see Figure 2.5) were cultured in normal Adipocyte Nutrition Medium (Promocell, UK) or Adipocyte Nutrition Medium supplemented with glucose (15 mM) and/or insulin (1 μ M) for 24 hours. This glucose concentration is similar to the serum glucose concentration that may be seen in uncontrolled human diabetes. The insulin concentration used in this study was considerably greater than physiological concentrations, but this was required to mimic hyperinsulinaemia, as the adipocytes were continuously cultured with a physiologically high concentration of insulin in order to maximise cell growth and differentiation.

6.3.3 Anandamide and 2-AG

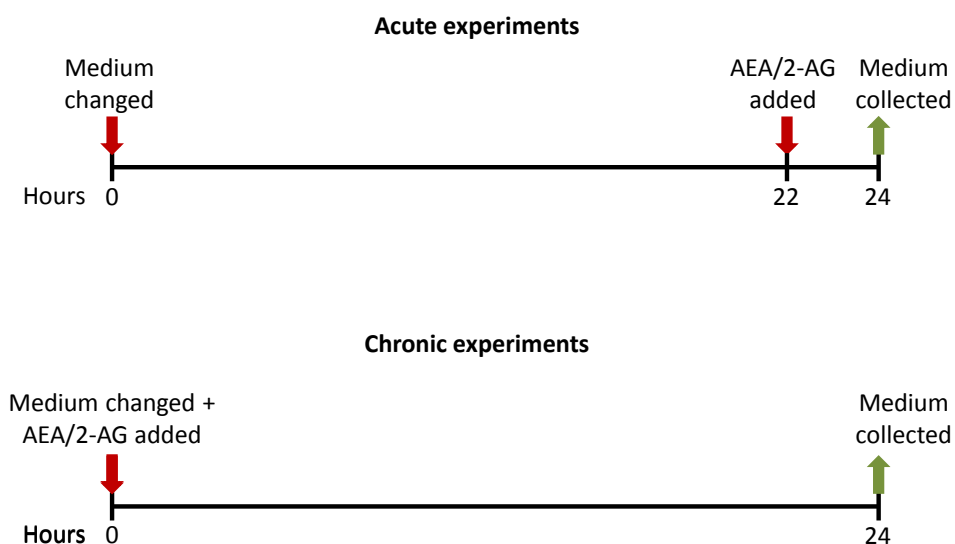
In the acute (2 hour) experiments varying concentrations of anandamide or 2-AG (final concentrations 10 nM to 10 μ M) were added to the plates at the same time as the medium was changed. In the chronic experiments (24 hours), anandamide or 2-AG was added 2 hours before the media were harvested (see Picture 6.1). In each plate, three wells were used for each concentration of endocannabinoid, with a further three wells for the vehicle control. The harvested media were immediately frozen and stored at -80°C for later analysis.

6.3.4 Adipokine assays

Commercially available sandwich ELISA sets (R&D) were used to measure the concentrations of adiponectin, leptin and resistin in the cell culture media (see Section 2.7.3).

6.3.5 Statistical analysis

All data were analysed using GraphPad Prism software. The adipokine concentrations from different culture conditions were compared using one-way ANOVA and Dunnett's multiple comparison test.



Picture 6.1 Timelines of cell culture experiments

6.4 Results

6.4.1 Effects of glucose and insulin

Adiponectin concentration was not affected by glucose, insulin or glucose and insulin combined at either 2 or 24 hours. Similarly, leptin secretion was not affected by glucose or insulin.

6.4.2 Acute endocannabinoid treatment and adiponectin

Treatment of differentiated adipocytes with anandamide for 2 hours did not affect adiponectin secretion compared to the vehicle control at any of the concentrations tested (10 nM to 10 μ M). This was true in adipocytes cultured in normal, high glucose (15 mM), high insulin (1 μ M) or high glucose and insulin media for 24 hours (Figure 6.1). Exposure to varying concentrations of 2-AG (10 nM to 10 μ M) for 2 hours also did not affect adiponectin secretion under normal, high glucose, high insulin or high glucose and insulin concentrations (Figure 6.2).

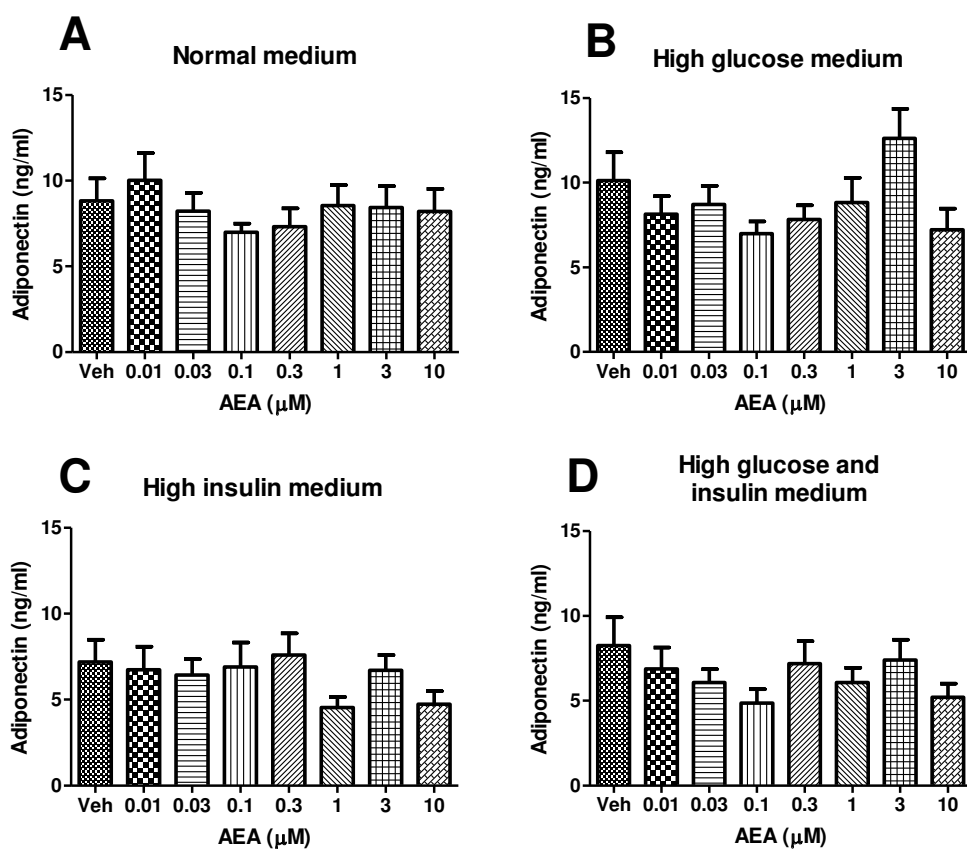


Figure 6.1 Effects of acute anandamide on adiponectin secretion

The effects of anandamide (2 hour treatment) on adiponectin secretion, in normal (A), high glucose (B), high insulin (C) and high glucose and insulin (D) media. $n=12$ (based on 4 experiments). Data are given as means, with error bars representing S.E.M., and were analysed using one-way ANOVA and Dunnett's multiple comparison test to compare different anandamide concentrations against vehicle.

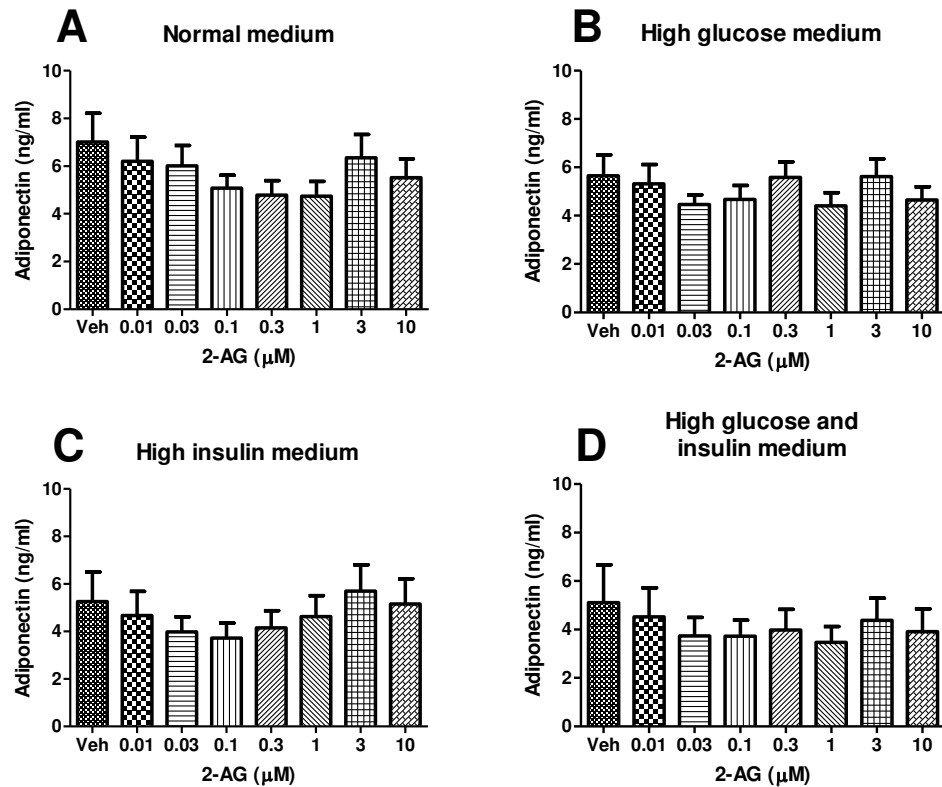


Figure 6.2 Effects of acute 2-AG on adiponectin secretion

The effects of 2-AG (2 hour treatment) on adiponectin secretion, in normal (A), high glucose (B), high insulin (C) and high glucose and insulin (D) media. $n=12$ (based on 4 experiments). Data are given as means, with error bars representing S.E.M., and were analysed using one-way ANOVA and Dunnett's multiple comparison test to compare different anandamide concentrations against vehicle.

6.4.3 Acute endocannabinoid treatment and leptin

Anandamide treatment for 2 hours, at increasing concentrations, did not affect the concentration of leptin in the adipocyte culture media. Glucose, insulin and combined glucose and insulin did not alter the leptin concentration, nor cause anandamide to have an effect on leptin secretion (Figure 6.3). Similarly, the acute 2-AG treatments did not affect the concentration of leptin in the media under any of the conditions tested (Figure 6.4).

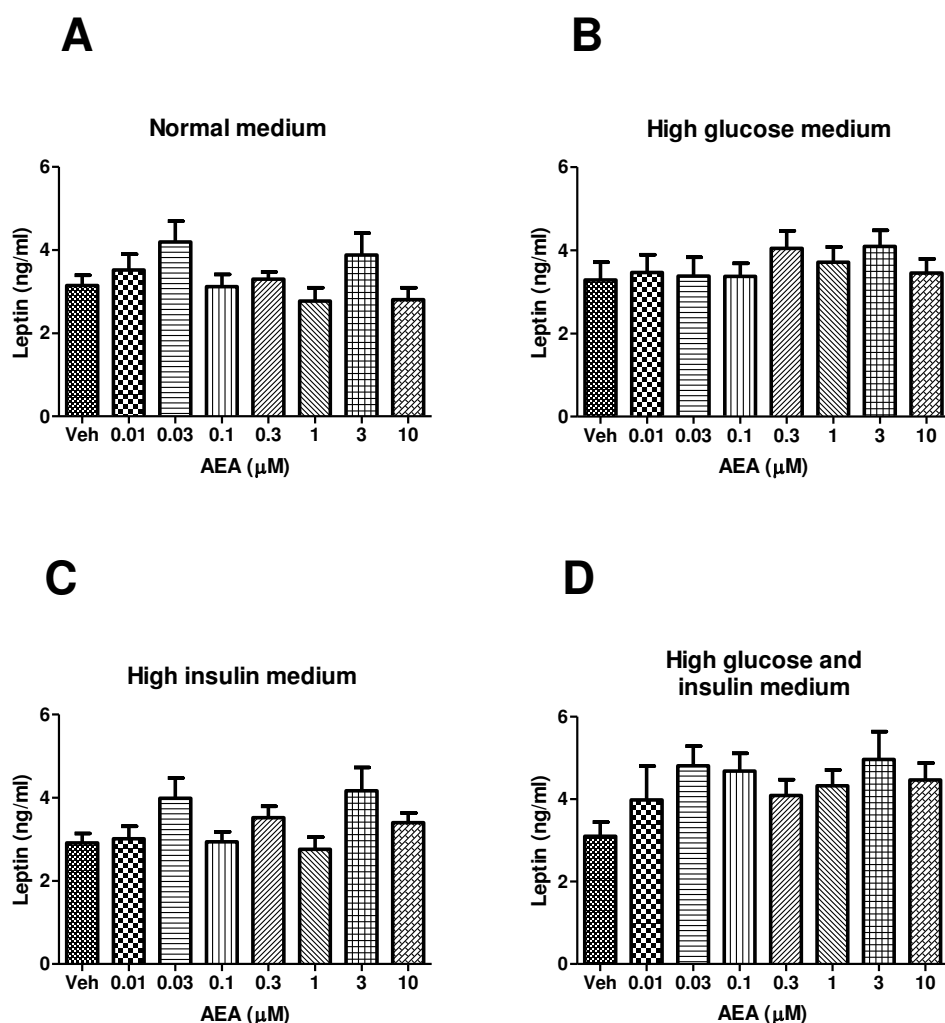


Figure 6.3 Effects of acute anandamide on leptin secretion

The effects of anandamide (2 hour treatment) on leptin secretion, in normal (A), high glucose (B), high insulin (C) and high glucose and insulin (D) media. $n=12$ (based on 4 experiments). Data are given as means, with error bars representing S.E.M., and were analysed using one-way ANOVA and Dunnett's multiple comparison test to compare different anandamide concentrations against vehicle.

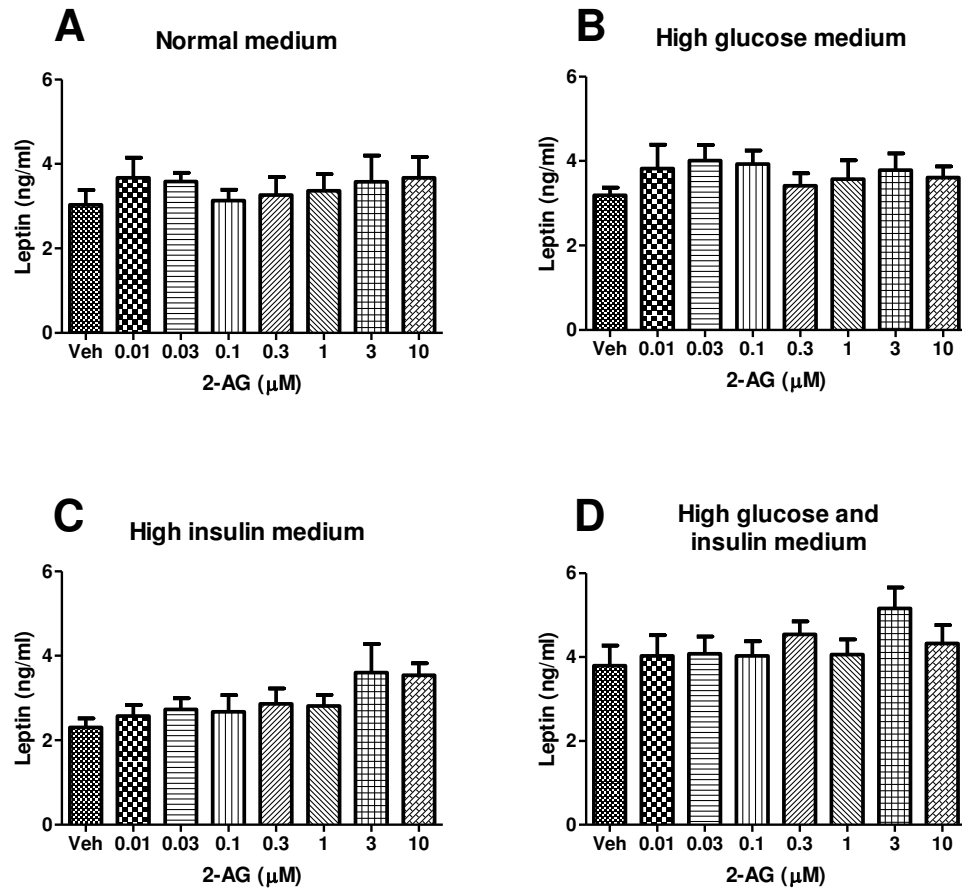


Figure 6.4 Effects of acute 2-AG on leptin secretion

The effects of 2-AG (2 hour treatment) on leptin secretion, in normal (A), high glucose (B), high insulin (C) and high glucose and insulin (D) media. $n=12$ (based on 4 experiments). Data are given as means, with error bars representing S.E.M., and were analysed using one-way ANOVA and Dunnett's multiple comparison test to compare different anandamide concentrations against vehicle.

6.4.4 Chronic endocannabinoid treatment and adiponectin

Adiponectin secretion by the cultured adipocytes was not affected by exposure to anandamide for 24 hours, or by glucose, insulin or glucose and insulin (Figure 6.5). Likewise, chronic 2-AG did not alter adiponectin secretion at any of the concentrations tested, even in the presence of high concentrations of glucose and/or insulin (Figure 6.6).

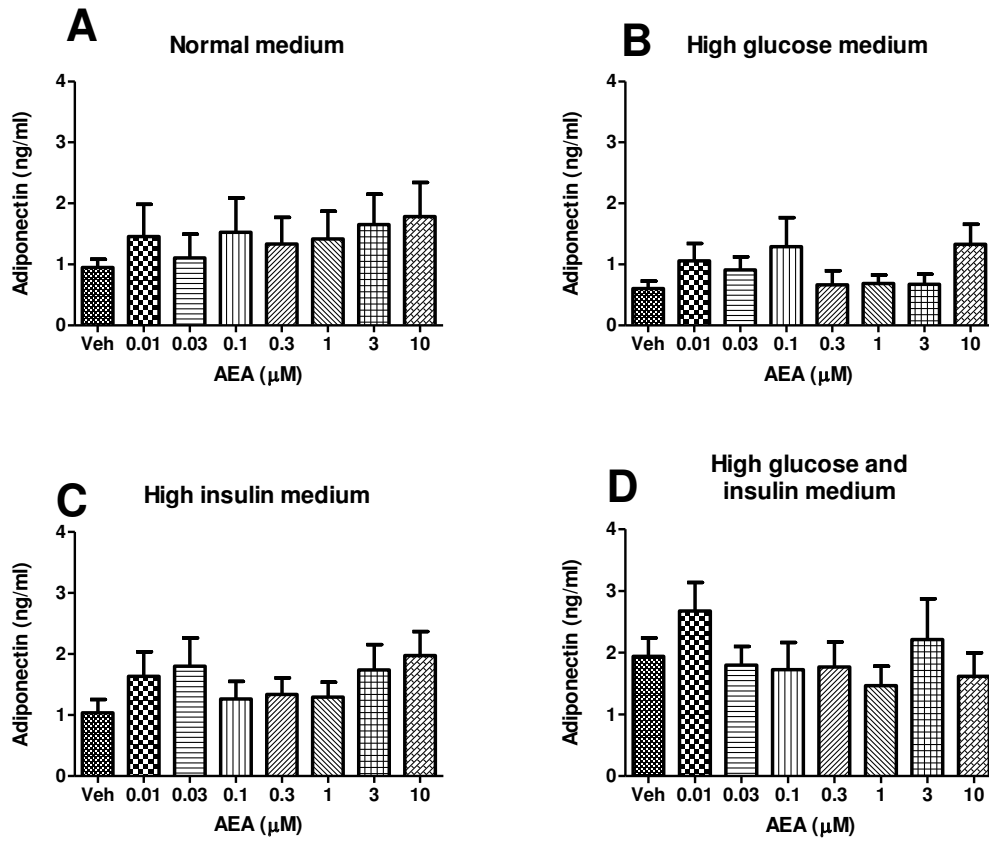


Figure 6.5 Effects of chronic anandamide on adiponectin secretion

The effects of anandamide (24 hour treatment) on adiponectin secretion, in normal (A), high glucose (B), high insulin (C) and high glucose and insulin (D) media. $n=12$ (based on 4 experiments). Data are given as means, with error bars representing S.E.M., and were analysed using one-way ANOVA and Dunnett's multiple comparison test to compare different anandamide concentrations against vehicle.

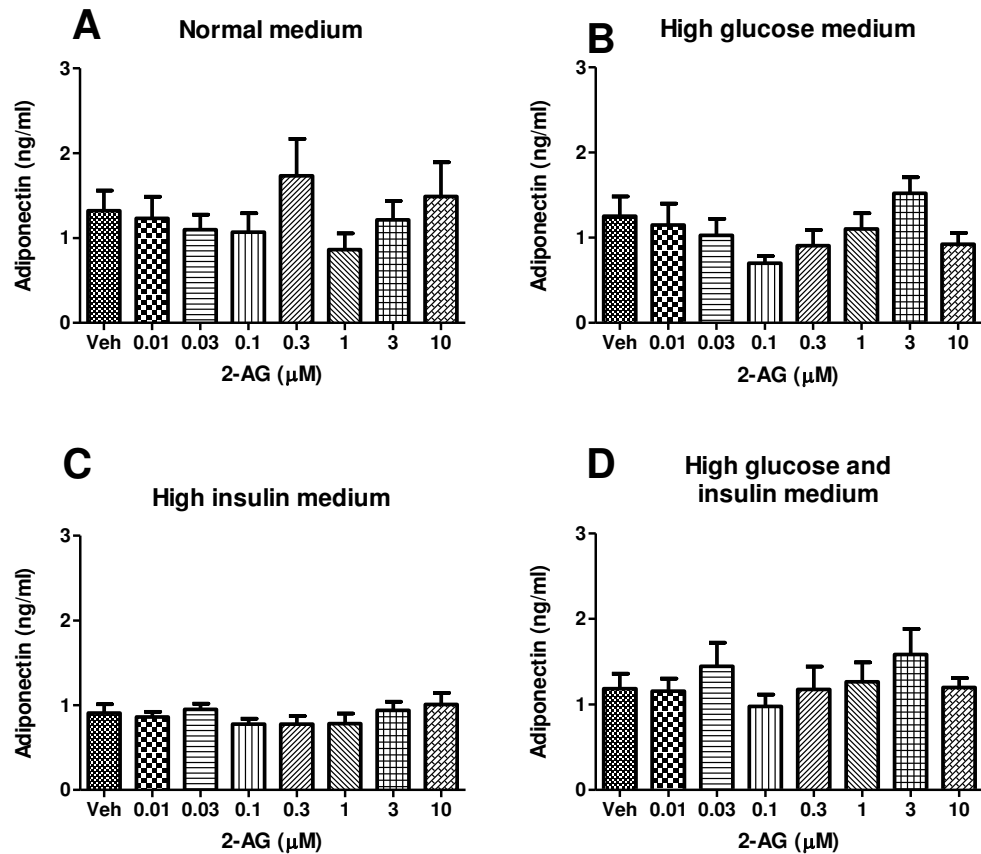


Figure 6.6 Effects of chronic 2-AG on adiponectin secretion

The effects of 2-AG (24 hour treatment) on adiponectin secretion, in normal (A), high glucose (B), high insulin (C) and high glucose and insulin (D) media. $n=12$ (based on 4 experiments). Data are given as means, with error bars representing S.E.M., and were analysed using one-way ANOVA and Dunnett's multiple comparison test to compare different anandamide concentrations against vehicle.

6.4.5 Chronic endocannabinoid treatment and leptin

Treatment of the cultured adipocytes for 24 hours with anandamide did not affect leptin secretion. Exposure to glucose, insulin and glucose and insulin in combination did not cause anandamide to have an effect on leptin (Figure 6.7). Similarly, the leptin concentration of the cell culture media was not altered in response to chronic 2-AG under basal, high glucose, high insulin or high glucose and insulin culture conditions (Figure 6.8).

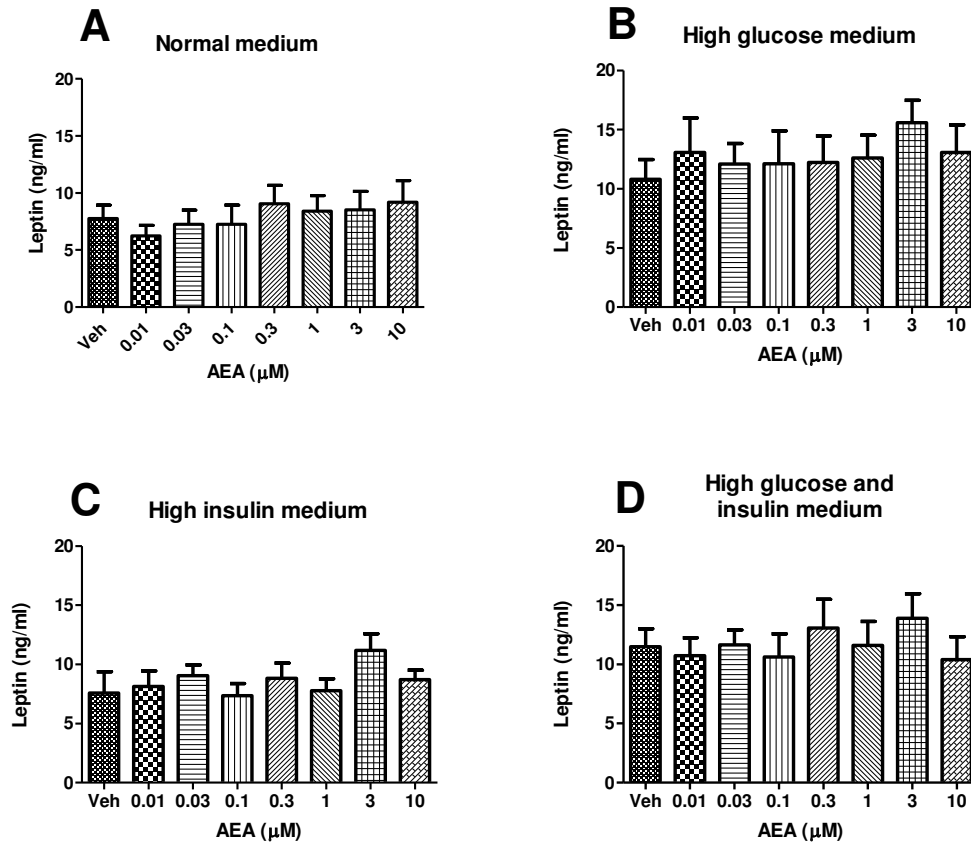


Figure 6.7 Effects of chronic anandamide on leptin secretion

The effects of anandamide (24 hour treatment) on leptin secretion, in normal (A), high glucose (B), high insulin (C) and high glucose and insulin (D) media. $n=12$ (based on 4 experiments). Data are given as means, with error bars representing S.E.M., and were analysed using one-way ANOVA and Dunnett's multiple comparison test to compare different anandamide concentrations against vehicle.

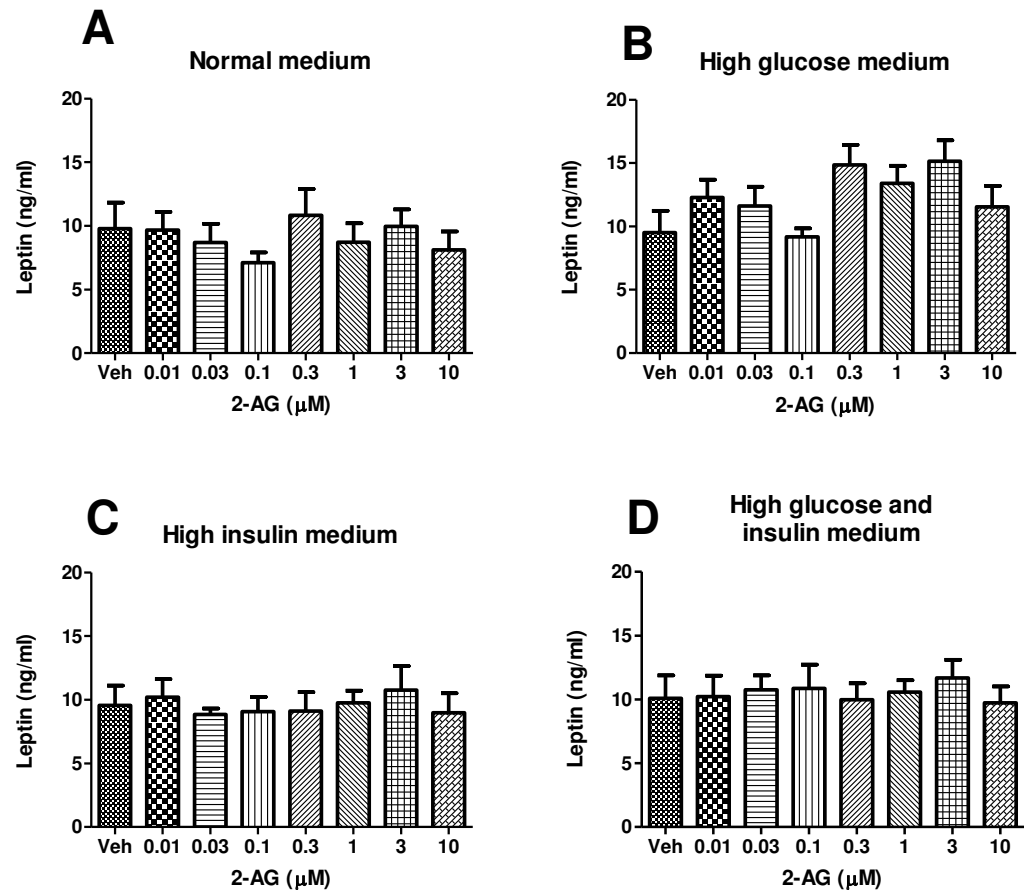


Figure 6.8 Effects of chronic 2-AG on leptin secretion

The effects of 2-AG (24 hour treatment) on leptin secretion, in normal (A), high glucose (B), high insulin (C) and high glucose and insulin (D) media. $n=12$ (based on 4 experiments). Data are given as means, with error bars representing S.E.M., and were analysed using one-way ANOVA and Dunnett's multiple comparison test to compare different anandamide concentrations against vehicle.

6.4.6 Resistin in cultured adipocytes

Resistin was not detectable in the media harvested from mature adipocytes cultured under any of the conditions described in this chapter. Similarly, the media harvested from preadipocyte cultures did not have detectable resistin concentrations. Treatment of the preadipocytes and differentiated adipocytes with anandamide and 2-AG did not induce resistin secretion.

6.5 Discussion

The results of Chapters 3-5 indicated that FAAH and MGL activities in mature adipocytes are not directly related to metabolic markers in rats or humans. However, there is other evidence that insulin and glucose affect the ECS in adipocytes. There is also a small amount of contradictory evidence that the ECS can affect adipokines and vice-versa, but this has not been extensively investigated in cultured human adipocytes. Therefore, the aim of this study was to investigate whether acute or chronic exposure to anandamide or 2-AG affects the secretion of adipokines from differentiated human adipocytes in culture under high insulin and/or glucose conditions. The results of this study showed that neither acute nor chronic anandamide or 2-AG treatments affect adipokine secretion in differentiated human adipocytes in culture. This was observed regardless of the glucose or insulin concentration of the treatment media.

There is some evidence that adipokines may regulate endocannabinoid levels. The most established theory is that leptin suppresses endocannabinoid levels at the central level, particularly in the hypothalamus (Di Marzo *et al.*, 2001). The effects of adipokines on endocannabinoids in adipocytes are not as clear. In human adipocytes in culture, leptin has been shown to decrease anandamide and 2-AG (Matias *et al.*, 2006). However, leptin and adiponectin have also been shown to have no effect on anandamide or 2-AG concentrations in cultured human adipocytes (Gonthier *et al.*, 2007). It has also been shown that ECS activation may affect adipokine expression, however adipokine secretion in response to anandamide and 2-AG has not yet been reported. In the results presented here, it is shown that anandamide and 2-AG do not affect the concentration of adiponectin or leptin in the adipocyte culture media after either 2 or 24 hours treatment. This was also found in the presence of excess glucose and insulin. It should be noted that the results from the two different timepoints cannot be directly compared, as different batches of cells were used for each. In the literature to date, there are no reports of the effects of anandamide or 2-AG on adipokine secretion in isolated adipocytes. Previously, PEA was reported to have no effect on adiponectin or leptin secretion from isolated human adipocytes, even at high concentrations (100 μ M) (Gonthier *et al.*, 2007). In humans, in the context of whole adipose tissue, it has been reported that CB₁ receptor mRNA is not correlated with adiponectin secretion, or its concentrations in adipose

tissue or blood (Lofgren *et al.*, 2007), which is in keeping with the cell culture results given in this chapter.

In this study, neither high glucose nor insulin affected the adipokine concentrations of the cell culture media. In recent years it has been widely reported that insulin increases the secretion of leptin from adipocytes, particularly rat adipocytes (reviewed by Lee and Fried, 2009). However, in earlier studies there were also reports of insulin having no effect on leptin in adipocytes (Considine *et al.*, 1997; Reul *et al.*, 1997). The reasons for these differences are not clearly apparent. To date the results reported for adiponectin are not conclusive. Insulin has been reported to increase adiponectin secretion or have no effect, and some of the differences have been attributed to the adipocytes used (3T3-L1, rat, human, omental or subcutaneous) (Motoshima *et al.*, 2002; Cong *et al.*, 2007). In the study presented here, the adipocytes were cultured for at least 5 weeks in commercial media containing higher than physiological concentrations of insulin. This was as recommended by the supplier of the adipocytes (Promocell) and has been reported elsewhere to increase the growth and differentiation rates of the adipocytes. Under the experimental conditions, the insulin concentration was increased further still, but it is possible that the adipocytes were partially insulin-resistant, which may account for the lack of adiponectin and leptin response to insulin. In support of this, it has been reported previously that primary culture of healthy rat adipocytes rapidly causes changes in gene expression patterns that are similar to the expression patterns seen in fresh adipocytes from obese Zucker rats (Xiang *et al.*, 2007).

Resistin secretion has been detected from adipose tissue explants (Fain *et al.*, 2003), and in isolated adipocytes and preadipocytes resistin mRNA and protein have also been reported (Kim *et al.*, 2001; Janke *et al.*, 2002; Degawa-Yamauchi *et al.*, 2003). However, several other studies have failed to detect resistin mRNA or protein in adipocytes (Nagaev and Smith, 2001; Savage *et al.*, 2001; Janke *et al.*, 2002; Fain *et al.*, 2003). The effects of insulin and glucose on resistin secretion in cultured human adipocytes have not been reported. Furthermore, to date, the effect of endocannabinoids on resistin have not been investigated. In this study it was found that resistin was not present in the cell culture media of adipocytes or preadipocytes under normal cell culture conditions. High concentrations of glucose and/or insulin did not stimulate resistin secretion after 24 hours, and short treatments (2 hour) with anandamide or 2-AG

did not induce resistin production. These findings are in keeping with several studies which have not detected resistin in cultured or fresh adipocytes (Nagaev and Smith, 2001; Savage *et al.*, 2001; Janke *et al.*, 2002; Fain *et al.*, 2003). In two studies in which resistin was detected in human adipocytes, either fresh adipocytes (Degawa-Yamauchi *et al.*, 2003) or primary adipocytes cultured for short time periods (48 hours) (McTernan *et al.*, 2003) were used. In 3T3-L1 cells resistin mRNA levels have been shown to be increased by glucose and decreased by insulin (Haugen *et al.*, 2001; Shojima *et al.*, 2002; Liu *et al.*, 2008), but this has not been replicated in human adipocytes. The results presented here show that glucose does not induce resistin production in human adipocytes. In addition, in this study the endocannabinoids anandamide and 2-AG did not cause resistin secretion. This suggests that there is not a direct relationship between the ECS and resistin in human preadipocytes or differentiated adipocytes. A recent review concluded that circulating resistin is predominantly from non-adipocytes (Schwartz and Lazar, 2011) and this is supported by the results of this chapter.

In summary, the effects of anandamide and 2-AG on adiponectin, leptin and resistin secretion by adipocytes have not been previously reported. In the novel study presented here it has been shown that these two endocannabinoids do not affect adiponectin, leptin or resistin secretion after 2 or 24 hours. Furthermore, glucose and insulin were not found to affect the action of anandamide or 2-AG on adipokine secretion. These results suggest that modulation of adipokines is unlikely to be an effect of endocannabinoids under normal conditions, or those associated with obesity and diabetes.

7. Endocannabinoid uptake in cultured human adipocytes

7.1 Introduction

The termination of endocannabinoid signalling is characterised by two key stages: cellular uptake of the endocannabinoids and subsequent enzymatic inactivation (Yates and Barker, 2009a). The mechanism(s) of endocannabinoid uptake have yet to be elucidated. There is evidence that anandamide uptake occurs by simple diffusion, which is plausible due to the lipophilic structure of the molecule (Glaser *et al.*, 2003). Alternatively, other data suggest that facilitated diffusion, possibly via the putative anandamide membrane transport (AMT) protein, or endocytosis is responsible (Di Marzo *et al.*, 2004; Yates and Barker, 2009b; Ligresti *et al.*, 2010). In support of this hypothesis it has been shown that anandamide uptake is time and temperature dependent, saturable and subject to inhibition by structural analogues of anandamide (Beltramo *et al.*, 1997; Hillard *et al.*, 1997). It may be that anandamide uptake occurs via both simple and facilitated diffusion, perhaps to differing degrees in different cell types or under different conditions. Whatever the mechanism, the uptake of anandamide in many cell types is at least partially regulated by the level of intracellular FAAH activity, as inhibition or deletion of FAAH has been shown to reduce anandamide uptake in several studies (Ortega-Gutierrez *et al.*, 2004; Kaczocha *et al.*, 2006). This indicates that in some systems anandamide uptake is dependent on maintenance of a concentration gradient.

Anandamide trafficking is still a relatively novel area of research, and as such has only been studied in a few cell types and systems. It is likely that binding proteins are required, as anandamide is a structurally simple molecule and is too lipophilic to exist unbound in the cytosol (for review see Maccarrone *et al.*, 2010). Several putative binding proteins have been proposed, including heat shock protein 70 (HSP70; Oddi *et al.*, 2009), albumin (Oddi *et al.*, 2009) and fatty acid binding proteins 5 and 7 (FABP5 and 7; Kaczocha *et al.*, 2009). The relevance of these proteins in anandamide trafficking in adipocytes is not yet known. Albumin is expressed by 3T3-L1 adipocytes (Yoo *et al.*, 2010) and HSP 70 is expressed in cultured rat adipocytes (Jiang *et al.*, 2007), but these proteins have not yet been described in human adipocytes. The FABPs are known to transport hydrophobic long chain fatty acids in the aqueous environment of the cytosol. It may be that FABP4 (the FABP expressed in

adipocytes) binds anandamide as FABP5 and 7 do (Kaczocha *et al.*, 2009), but this awaits confirmation.

Adiposomes are lipid droplets surrounded by protein which are found in most cells, but they are particularly pronounced in differentiating and mature adipocytes. It has been demonstrated *in vitro* that the rate of anandamide uptake increases with adiposome size and that anandamide readily accumulates at adiposomes (Oddi *et al.*, 2008). Biotin and tritium labelled anandamide have been used to study the intracellular distribution of anandamide and it has been found that the majority of anandamide is incorporated into intracellular membranes within minutes of uptake (Oddi *et al.*, 2008; Oddi *et al.*, 2009). The implications of this for anandamide uptake and trafficking in adipocytes have yet to be investigated. Given the role and physiological nature of adipocytes and the lipophilic properties of anandamide, it may be that different trafficking systems are involved in mature adipocytes compared to other cells.

Anandamide uptake has been reported in 3T3-L1 adipocytes (Gasperi *et al.*, 2007), but no published work to date has described anandamide uptake in human adipocytes. In 3T3-L1 cells, anandamide uptake was reported to be AMT mediated, as uptake was saturable, significantly increased at 37°C compared to 4°C and reduced by OMDM1 (a putative selective AMT inhibitor). Anandamide uptake, reported as AMT activity, was approximately double at 9 days after induction of differentiation compared with day 0. These data are in keeping with many of the studies described in Section 1.4 showing that the ECS becomes activated during adipocyte differentiation.

The cellular uptake of 2-AG has been investigated less than that of anandamide. In common with anandamide uptake, the mechanisms of 2-AG uptake remain under debate, although it has been suggested that anandamide and 2-AG uptake may rely on the same transport system (for review see Hermann *et al.*, 2006). In one study in which 2-AG uptake has been reported, the non-specific enzyme inhibitor MAFP did not reduce 2-AG uptake in several of the cell types assayed (Fowler and Ghafouri, 2008). This is in contrast to anandamide uptake which is generally more susceptible to reduction via FAAH inhibition. The authors of this work suggest that 2-AG may undergo transformation by other enzymes, such as acyltransferases or kinases, which have not yet been investigated with regard to 2-AG uptake (Fowler and Ghafouri, 2008).

One of the major findings of all the studies into endocannabinoid uptake is that it varies widely between different cell types. Not only does the rate of uptake vary between cells, but inhibition of enzymes which catalyse endocannabinoid degradation causes reductions in endocannabinoid uptake in some cells but not others (Thors *et al.*, 2007; Fowler and Ghafouri, 2008). To date there are no data available on the contribution of FAAH and MGL to anandamide and 2-AG uptake in human adipocytes. In Chapters 3 and 5 evidence from the current literature was discussed which suggested possible relationships between insulin, glucose and FAAH and MGL expression. The effects of insulin and glucose on FAAH and MGL activities have not been reported in cultured human adipocytes. In the current chapter, the enzyme activities were measured under the same conditions as the endocannabinoid uptake studies in order to determine whether insulin and glucose affect these two stages of endocannabinoid inactivation.

There is mounting evidence that the endocannabinoids anandamide and 2-AG affect various aspects of metabolism in adipocytes, including glucose uptake, lipolysis and lipogenesis (see Section 1.5). By contrast, there are very few data on the effects of insulin and glucose on the ECS in adipocytes. Investigating whether insulin or glucose affect anandamide or 2AG uptake will contribute to the understanding of the role of the ECS in adipocytes.

7.2 Aims

The uptake of endocannabinoids is an essential step of their inactivation by FAAH and MGL, but as yet this has not been described in human adipocytes. This study was based on the current literature showing that the ECS in adipocytes in health is not fully characterised, and that alterations in the ECS in adipose tissue have been reported in metabolic disorders. Therefore, the primary aim of this study was to investigate whether the uptake of anandamide or 2-AG in human adipocytes is affected by chronic or acute treatment with high concentrations of glucose or insulin. In this study, specific FAAH and MGL inhibitors had no effect on endocannabinoid uptake, so the second aim was to assess the effect of insulin and glucose treatments on FAAH and MGL activities. This also allowed the effects of insulin and glucose on FAAH and MGL to be investigated in a controlled, uniform study to complement the work of Chapters 3-5.

7.3 Materials and Methods

7.3.1 Endocannabinoid uptake experiments

This work was carried out at Lund University, Sweden, under the supervision of Professor Christopher Fowler (Fowler *et al.*, 2004; Fowler, 2006; Fowler and Ghafouri, 2008). Cultured human adipocytes (see Section 2.8.1) and the anandamide and 2-AG uptake assays (see Section 2.8.4) were used in these experiments. The endocannabinoid uptake assays are well established techniques within Professor Fowler's team. Glucose (15 mM) and insulin (1 μ M), when used, were added to the plates for 2 or 24 hours. After the first washing stage, 10 μ l of vehicle (DMSO), URB597 (final concentration 1 μ M) or JZL184 (final concentration 1 μ M) were added to the plates.

7.3.2 FAAH and MGL activity experiments

The FAAH and MGL activity assays were performed as described in Section 2.5. The adipocytes for these experiments were cultured in 6-well plates, as described in Section 2.8.1, and treated with glucose (15 mM) and/or insulin (1 μ M) for 2 or 24 hours. Following the treatment period, the cell culture medium was removed and 1 ml of TE buffer was added to each well. The plates were frozen at -80°C and subsequently thawed and re-frozen twice in order to lyse the adipocytes. After the third freeze-thaw cycle, the cells from each well were collected in the TE buffer using a cell scraper, transferred to tubes and stored at -80°C until enzyme activity analysis and protein assay. The cell lysates were used in the FAAH and MGL assays without further dilution. Each sample was run in duplicate.

7.3.3 Statistical analysis

All data were analysed using GraphPad Prism software. The enzyme activities from different culture conditions were compared using one-way ANOVA and Dunnett's multiple comparison test.

7.4 Results

7.4.1 Anandamide uptake

In differentiated human adipocytes, acute exposure to insulin (1 μ M), glucose (15 mM) or both for 2 hours did not affect anandamide uptake (Figure 7.1A). In the chronic experiments, exposure to insulin for 24 hours led to a significant increase in total anandamide uptake (1.2 ± 0.2 vs 1.8 ± 0.2 pmoles). Statistically, this effect was ameliorated by the presence of glucose (Figure 7.1B). There were no differences in anandamide uptake between cells cultured with glucose or insulin and glucose for 2 hours compared to those exposed to the conditions for 24 hours (Figure 7.1A,B).

7.4.2 FAAH inhibition and anandamide uptake

Preincubation with URB597, a FAAH inhibitor, did not affect basal anandamide uptake over the 4 minute period studied here (Figure 7.1C,D). Additionally, FAAH inhibition did not alter the amount of anandamide uptake in the acute or chronic experiments with insulin and glucose. Furthermore, insulin-stimulated anandamide uptake was not reduced by FAAH inhibition (1.1 ± 0.2 vs 1.9 ± 0.2 pmol; Figure 7.1D).

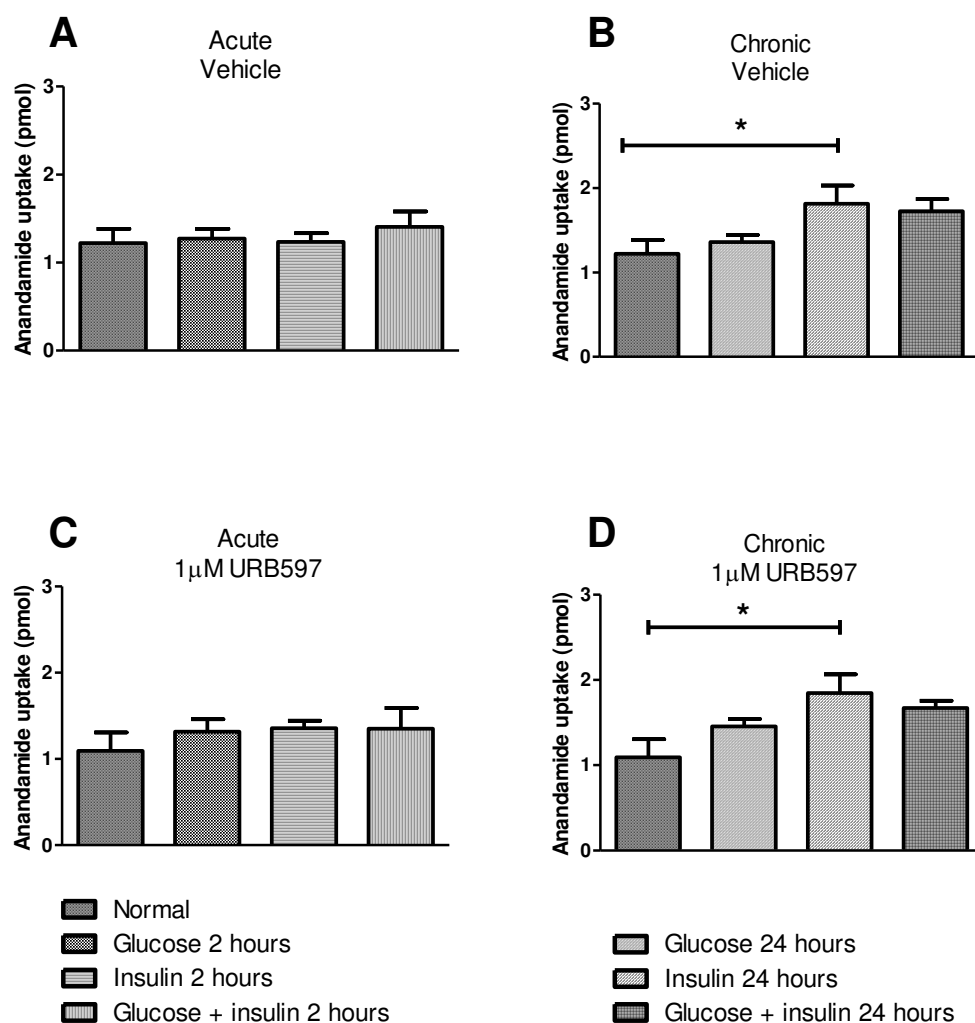


Figure 7.1 Anandamide uptake

Anandamide uptake in human adipocytes cultured in the absence and presence of glucose (15 mM) and insulin (1 μ M) for 2 or 24 hours. Results shown for vehicle after acute (A) and chronic (B) exposure, and using the FAAH inhibitor URB597 after acute (C) and chronic (D) exposure. Data were analysed by one-way ANOVA and Dunnett's multiple comparison test (* $P < 0.05$). $n = 4$ (in triplicate).

7.4.3 2-AG uptake

The basal level of 2-AG uptake in differentiated human adipocytes was not affected by insulin, glucose or both in combination at either the 2 or 24 hour time point (Fig. 7.2A,B).

7.4.4 MGL inhibition and 2-AG uptake

Inhibition of MGL, using the specific irreversible MGL inhibitor JZL184, did not alter 2-AG uptake by adipocytes in any of the conditions investigated (Figure 7.2C,D).

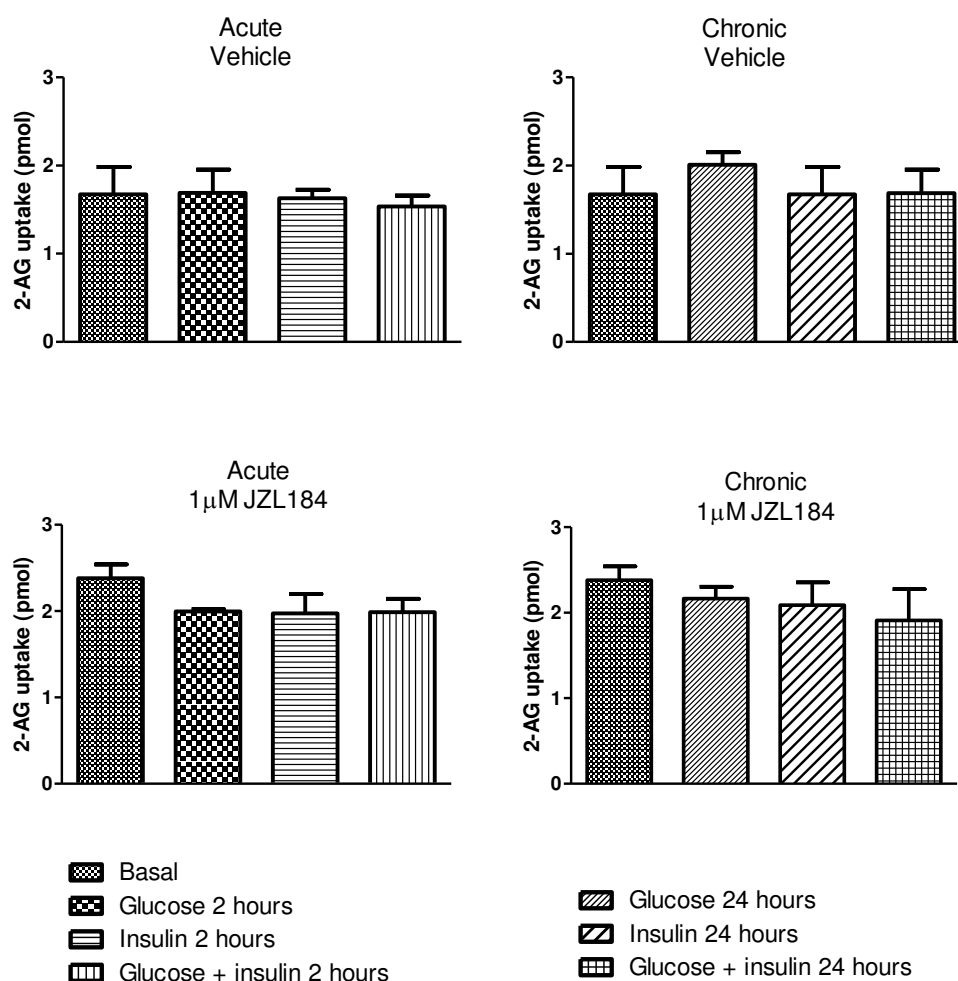


Figure 7.2 2-AG uptake

2-AG uptake in human adipocytes cultured in the absence and presence of glucose (15 mM) and insulin (1 µM) for 2 or 24 hours. Results shown for vehicle after acute (A) and chronic (B) exposure, and using the FAAH inhibitor URB597 after acute (C) and chronic (D) exposure. Data were analysed by one-way ANOVA and Dunnett's multiple comparison test (* $P < 0.05$). $n = 4$ (in triplicate).

7.4.5 Enzyme activity

The finding that URB597 did not affect anandamide uptake was supported by the finding that FAAH activity is negligible in human adipocytes cultured under the conditions described here. FAAH activity was not increased to detectable levels in adipocytes cultured with either insulin or glucose. By contrast, MGL activity was detectable in cultured adipocytes at a basal level of 7.8 ± 0.4 nmoles.min⁻¹.mg protein⁻¹ (Figure 7.3).

Exposure to glucose for 2 hours did not affect MGL activity (6.4 ± 0.2 nmoles.min⁻¹.mg protein⁻¹), but after 24 hours glucose decreased MGL

activity (5.9 ± 0.1 nmoles.min⁻¹.mg protein⁻¹, $P < 0.01$). Insulin decreased MGL activity at 2 and 24 hours (5.8 ± 0.7 and 5.0 ± 0.4 nmoles.min⁻¹.mg protein⁻¹ respectively, $P < 0.01$ and $P < 0.001$), and glucose and insulin in combination also reduced MGL activity at 2 and 24 hours (5.1 ± 0.4 and 3.1 ± 0.3 nmoles.min⁻¹.mg protein⁻¹ respectively, $P < 0.001$).

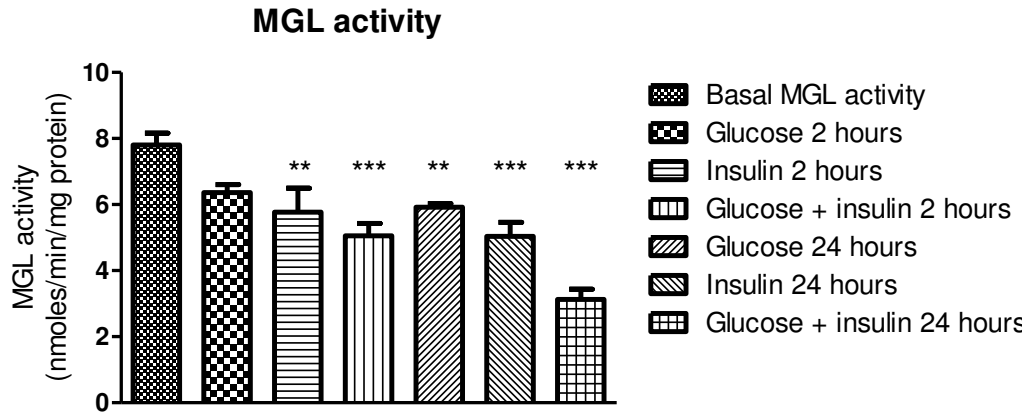


Figure 7.3 MGL activity

MGL activity in human adipocytes cultured in the absence and presence of glucose (15 mM) and insulin (1 μ M) for 2 or 24 hours. Data were analysed by one-way ANOVA and Dunnett's multiple comparison test to compare basal activity against all treatments (** $P < 0.01$, *** $P < 0.001$). $n = 4$ (in triplicate).

7.5 Discussion

The principal aim of this study was to determine whether the uptake of anandamide or 2-AG by differentiated human adipocytes is affected by acute or chronic exposure to high concentrations of insulin or glucose. The results presented here show that anandamide uptake is increased in adipocytes which have been exposed to insulin for 24 hours, but not under any of the other conditions tested. 2-AG uptake is not affected by insulin or glucose. Furthermore, anandamide and 2-AG uptake by adipocytes cultured under the conditions described in this study are not FAAH or MGL dependent. As anandamide and 2-AG uptake were not affected by enzyme inhibition, the secondary aim of the study was to measure FAAH and MGL activities in these cells to determine whether they are affected by glucose or insulin. FAAH activity was not detectable in the human adipocytes used here, but MGL activity was suppressed by high concentrations of insulin and glucose.

The concentration of anandamide in subcutaneous adipose tissue has been reported to be increased in obese diabetic humans, compared to lean and obese non-diabetic subjects (Annuzzi *et al.*, 2010). Furthermore, in 3T3-L1 cells 24 hour exposure to insulin decreases the intracellular concentration of anandamide by approximately a third (D'eon *et al.*, 2008). These findings suggest that glucose or insulin may affect the regulation of anandamide levels in adipocytes, but the mechanisms behind this have not been extensively investigated to date. In particular, the effects of insulin and glucose on anandamide uptake have not been reported. The results presented in the current study show, for the first time, that 24 hour exposure to a high insulin concentration (1 μ M) increases the rate of anandamide uptake in human adipocytes. The two potential effects of this would be to reduce extracellular signalling, such as at the cannabinoid receptors, and to increase intracellular signalling at targets such as the PPARs and TRPs. One possible reason for increased anandamide uptake is suggested by the findings that anandamide increases the rate of PPAR γ mediated differentiation of 3T3-L1 cells (Bouaboula *et al.*, 2005) and rat adipocytes (Karaliota *et al.*, 2009). In a state of chronic hyperinsulinaemia, increased anandamide uptake may be one of the mechanisms for increasing the number of mature adipocytes, and therefore the glucose and lipid storage capacity of adipose tissue.

It is interesting to note that in 3T3-L1 cells anandamide has been shown to increase the rate of insulin-stimulated glucose uptake after 24

hours, but not at 4 hours (Gasperi *et al.*, 2007). Taken with the results presented here, this finding suggests interactions between anandamide and glucose uptake in response to insulin only after prolonged exposure. This may indicate that anandamide concentration and signalling is reflective of metabolic balance in the medium and long-term rather than acute.

In many cell types, it has been shown that anandamide uptake is at least partially driven by FAAH and the maintenance of an anandamide concentration gradient between the cytosol and extracellular space (Thors *et al.*, 2007). There are also reports of cells in which anandamide uptake is not dependent on the rate of anandamide hydrolysis (Di Marzo *et al.*, 1998), but no data have been published on the contribution of FAAH to anandamide uptake in adipocytes. Having showed that anandamide uptake was not URB597 (FAAH) dependent, the presence and activity of FAAH was investigated in these cells. The results presented in the current study show that FAAH activity is not detectable in cultured human adipocytes. By contrast, in a study using cultured 3T3-L1 cells FAAH activity was reported, and found to be higher in differentiated adipocytes than the fibroblasts (Gasperi *et al.*, 2007). It may be that 3T3-L1s genuinely have higher FAAH expression than human adipocytes in culture, or that differences in the culture medium affected FAAH expression. For example, the 3T3-L1s were cultured without insulin for 3-5 days before FAAH activity was measured, whereas the differentiated adipocytes used in the study described here were cultured with insulin constantly, as this is a component of the adipocyte medium. The absence of appreciable FAAH activity in this study explains why the specific FAAH inhibitor URB597 did not affect anandamide uptake. Given that FAAH activity was not detected, it is not possible to comment on whether anandamide uptake has a FAAH dependent component *in vivo* in human adipocytes, as in the mature adipocytes isolated from human biopsies (Chapters 4 and 5) FAAH activity was detected. Several differences between freshly isolated and cultured adipocytes have been reported. One obvious difference is that cultured human adipocytes which are differentiated *in vitro* are morphologically very different from freshly isolated mature adipocytes. The environment of cell culture is clearly very different from the *in vivo* environment, and it may be that the ECS is not highly expressed in cultured adipocytes compared to fresh adipocytes. In future studies it might prove informative

to measure anandamide uptake in mature adipocytes isolated from adipose tissue biopsies.

2-AG uptake has not been investigated in adipocytes, but other fatty acids have been reported on. Several studies have shown that fatty acid uptake by adipocytes can be stimulated by insulin (Stump *et al.*, 2001; Stahl *et al.*, 2002; Varlamov *et al.*, 2010). In the current study, neither glucose nor insulin was found to affect 2-AG uptake in cultured human adipocytes. This indicates that insulin and glucose do not affect the ECS through alterations in the extracellular signalling of 2-AG. Fatty acid uptake by adipocytes has been suggested to occur through two distinct mechanisms: a non-saturable passive process and a saturable active transport system (Stump *et al.*, 2001). It is the active transport of fatty acids that is thought to be regulated by insulin (Varlamov *et al.*, 2010). The results of this study suggest that insulin does not significantly affect any proteins which may be involved in 2-AG transport.

In previous Chapters (3-5) of this thesis it has been shown that there is no direct correlation between MGL activity in adipocytes and blood plasma glucose and insulin concentrations. However, insulin has been shown to decrease intracellular 2-AG concentration and increase MGL mRNA levels in differentiated 3T3-L1 adipocytes (D'eon *et al.*, 2008). Additionally, the concentration of 2-AG in subcutaneous adipose tissue has been shown to be decreased in obese diabetic humans compared to lean and obese non-diabetic subjects (Annuzzi *et al.*, 2010). The data from the current study show that MGL activity in cultured human adipocytes is reduced by both acute and chronic treatment with insulin, with or without glucose. Furthermore, glucose alone decreases MGL activity after 24 hours. These data are at odds with the study which shows MGL mRNA in 3T3-L1 cells to be increased in response to insulin (D'eon *et al.*, 2008). This may reflect a difference between human adipocytes and the murine fibroblast cell line, or between mRNA and final protein activity. Other studies have shown adipose tissue 2-AG concentrations to be decreased in obese diabetic humans (Annuzzi *et al.*, 2010) and rats (Starowicz *et al.*, 2008), which is in keeping with the results observed in this chapter. One of the effects of insulin in adipocytes is to increase the rate of lipogenesis. From a metabolic perspective, it is reasonable that insulin and glucose should decrease MGL activity in adipocytes, as MGL is generally involved in lipolysis. However, this finding does contradict the widely held view that

MGL is present in abundance and does not fluctuate under the influence of hormones (Mead *et al.*, 2002).

In this study insulin and glucose did not affect 2-AG uptake, despite altering MGL activity. This provides further evidence, in addition to the lack of effect of the MGL inhibitor JZL184, that 2-AG uptake in human adipocytes is MGL independent. This has also been reported in several other cell types (Fowler and Ghafouri, 2008).

This study has provided evidence that anandamide uptake in human adipocytes increases in response to chronic insulin exposure, but is not affected by acute treatment or glucose. By contrast, 2-AG uptake was not affected by acute or chronic insulin or glucose, but MGL activity was suppressed by both insulin and glucose. In contrast to some other cell types, anandamide and 2-AG uptake in this study were not FAAH or MGL dependent, suggesting that concentration gradients may not be important for endocannabinoid uptake in human adipocytes. FAAH activity was not detected in these cultured adipocytes, indicating that ECS signalling is markedly affected by culturing adipocytes for extended time periods. MGL activity was detected in these adipocytes, presumably reflecting its role in lipid metabolism. The differences observed in the uptake profiles of anandamide and 2-AG suggest that there is at least some proportion of their uptake that is dependent on specific transport proteins, which, in the case of anandamide, may be sensitive to insulin.

8. General discussion

In recent years, a large number of studies have identified the ECS as a potentially important regulatory system in metabolic homeostasis, both in the CNS and peripheral organs associated with metabolism. In adipose tissue, many of the results published to date have been contradictory and raised questions about the expression, role and regulation of the ECS in adipose tissue, particularly in obesity and diabetes. Furthermore, although there are several studies in which ECS proteins have been investigated in whole adipose tissue samples, there are very few reports on the ECS in isolated mature adipocytes. Therefore, the overall aim of this thesis was to further understand the role of the ECS in adipocytes, and how this might be affected by obesity or metabolic dysfunction.

In the first phase of this project, three strains of Zucker rat were used as models of lean, obese and obese diabetic states, as some aspects of the ECS have been found to be similar between these rat models and human studies (for recent reviews see Andre and Gonthier, 2010; Silvestri *et al.*, 2011). The major novel findings of this study were that both FAAH and MGL activities in mature adipocytes were correlated with body weight. A review of FAAH relevant to this study proposed that "endocannabinoid signaling is under the tonic control of FAAH in vivo" (McKinney and Cravatt, 2005). If this is true in adipocytes, it suggests that the upregulation of FAAH activity seen in the obese rats may significantly alter the overall expression and signalling of the ECS in adipocytes.

Previous studies have shown differences in FAAH and MGL mRNA expression between subcutaneous and visceral adipose tissue in humans (Blüher *et al.*, 2006; Pagano *et al.*, 2007), so adipocytes from two visceral and one subcutaneous adipose tissue depot were investigated in the Zucker rats. It was found that FAAH and MGL activities were generally not different between visceral and subcutaneous adipocytes. This shows that, even in obesity and diabetes, endocannabinoid degradation does not differ significantly between visceral and subcutaneous adipocytes.

In light of the data obtained in the Zucker rats, similar studies were conducted in humans. The first of which was based on metabolically healthy volunteers representing a range of BMIs (Chapter 4), and with normal glucose and insulin blood concentrations. This design allowed the effects of obesity (fat accumulation) on FAAH and MGL to be assessed, with no interference from co-morbidities such as diabetes. In this study FAAH activity in adipocytes was found to correlate with BMI, which is in

keeping with the results seen in the Zucker rats. FAAH activity also correlated with waist circumference, but not with other anthropometric measurements. Together, this suggests a strong relationship between visceral adiposity and endocannabinoid degradation in adipocytes. However, in this human study, MGL activity did not correlate with any of the variables measured, indicating that 2-AG degradation in adipocytes is not affected by obesity. This is in contrast to the correlation with weight seen in the Zucker rats, and may be evidence of differing roles or regulation of MGL between species, as discussed later.

The ECS may be dysregulated in diabetes, as blood concentrations of the endocannabinoids are increased (Matias *et al.*, 2006), and endocannabinoids levels are altered in adipose tissue (Annuzzi *et al.*, 2010). In the present research, in Zucker rats, differences in FAAH and MGL activities were observed between the three metabolic groups (healthy, obese and obese diabetic). This was not further investigated in the healthy humans (Chapter 4), as the study was specifically designed to exclude metabolic disorders. Therefore, the effect of metabolic disease on FAAH and MGL in adipocytes was addressed in Chapter 5, using obese patients with a spectrum of metabolic dysfunction. In this obese sample, FAAH and MGL activities in adipocytes were not altered in relation to anthropometric estimates of adiposity, or blood serum markers of metabolic homeostasis. Taken with the results of Chapter 4, showing a correlation between FAAH activity and BMI, this suggests that obesity affects anandamide degradation in adipocytes, but that none of the individual factors often associated with obesity appears to be driving this FAAH upregulation. A noticeable similarity between the Zucker rat and obese patient studies is that FAAH and MGL activities were similar between adipose tissue depots (visceral or subcutaneous). This is in contrast to studies which have shown FAAH mRNA to be increased (Blüher *et al.*, 2006) and MGL mRNA to be decreased (Pagano *et al.*, 2007) in visceral adipose tissue compared to subcutaneous adipose tissue. Given that in the studies presented here there is no difference between depots in rats or humans, the differences seen in the mRNA studies may highlight the value of measuring enzyme activity rather than gene transcripts. This may be particularly important as studies on post-transcriptional modifications of FAAH and MGL have not been published to date.

In the Zucker rats, FAAH activity was higher in the obese rats than in the obese diabetic rats, but this was not observed in the obese patients.

This difference may have arisen from the use of discrete rat strains, compared to the relatively continuous human data, or because the obese rats had a higher mean weight than the obese diabetic rats (which was not seen in the humans). It is also important to note that many of the patients were using diet and/or medication to try to control their blood glucose concentration, whereas the rats' diabetes was uncontrolled. Another difference between the Zucker rats and humans that may be important is that the maximum FAAH activities seen in the human adipocytes were much higher than the maximum activities measured in the rat adipocytes. Conversely, for MGL this relationship was reversed. In regards to MGL, similar results have been reported previously in experiments using cultured adipose tissue explants. It was found that the rate of stimulated lipolysis (involving MGL) was higher in animal samples than in human (reviewed by Arner, 1988). This suggests that there are differences in the roles of FAAH and MGL between rats and humans, and if so, data on the ECS (particularly endocannabinoid catabolism) collected from rat studies should be interpreted with some caution when extrapolations and comparisons to human data are made.

Various studies have demonstrated that there may be relationships between metabolic function and the ECS in adipocytes, but many of the specific aspects of these relationships remain unexplored. In particular, the effects of anandamide and 2-AG on adipokine secretion have not been reported in the literature. In Chapter 6, this concept was investigated under normal conditions, and high glucose and/or insulin conditions. This study found that the endocannabinoids did not affect adiponectin or leptin secretion, under any of the conditions tested. This study therefore strongly suggests that the endocannabinoids do not affect metabolic function via direct regulation of adipokine secretion.

In order to affect metabolic pathways via intracellular targets, the endocannabinoids first have to get into adipocytes. Endocannabinoid uptake, and the factors which may drive it, have been investigated in several cell types, but not in human adipocytes. In the context of the present research, it was considered important to establish whether insulin or glucose may affect endocannabinoid uptake. The human adipocyte cell culture model used in Chapters 6 and 7 also represented a convenient way to measure the effect of high insulin and/or glucose on FAAH and MGL activities to support our animal and human data. It was found that chronic exposure to insulin increased anandamide uptake. Taken with the results

of Chapter 4, showing increased FAAH activity in obesity, this suggests that in obese humans (who tend to have higher serum insulin concentrations than lean people), both anandamide uptake and degradation may be increased. In another human study, it was found that lean and obese humans have similar concentrations of anandamide in their subcutaneous adipose tissue (Annuzzi *et al.*, 2010). If both uptake (driven by insulin) and degradation of anandamide are increased in this obese state, this suggests that production of anandamide may also be increased in adipose tissue in obesity. This supports the concept of a general upregulation of the ECS in obesity. Interestingly, in obese, diabetic humans anandamide levels were reported to be increased in adipose tissue (Annuzzi *et al.*, 2010). According to the model suggested above, this could be mediated by a lack of insulin-stimulated anandamide uptake by insulin resistant adipocytes.

In Chapter 7, 2-AG uptake was not affected by insulin or glucose. Additionally, the results of Chapters 4, 5 and 6 show that MGL activity does not appear to have relationships with obesity, metabolic dysfunction or adipokine secretion. Together, these suggest that 2-AG signalling is not a key regulator of adipocytes. Considering the high concentration of intracellular 2-AG and high activity of MGL in adipocytes, and their roles in lipid metabolism, it might be surprising if 2-AG and MGL were also tasked with specific regulatory roles. Insulin has been shown to downregulate the rate of lipolysis (Duncan *et al.*, 2007), and a similar relationship was seen in the cell culture study (Chapter 7), in which insulin decreased MGL activity. Intriguingly, this finding does question the widely held tenet that MGL is not a rate-limiting step in lipolysis and is not under hormonal regulation. It may be that other enzymes in the lipolytic pathway are downregulated to a greater extent than MGL, and that therefore MGL activity remains relatively high, but further experiments would be required to investigate this. The focus of this thesis however is the ECS in adipocytes, and the conclusion from Chapter 7 is that the rate of 2-AG catabolism is increased by insulin, and by chronic glucose. It is important to consider this finding in light of the results of Chapters 3-5. In the hyperinsulinaemic Zucker rats, MGL activity was increased, and in the humans MGL activity did not show any relationship with insulin or metabolic status. These findings suggest that there are other factors *in vivo* which have a greater influence on MGL activity than insulin. It might also be that insulin resistance in the obese rats and humans had some

effect in altering the signalling between insulin and MGL. The use of insulin resistant adipocytes in culture could provide further insight in this complicated area.

8.1 Limitations and future work

One of the key aspects of this project was to investigate the ECS in isolated mature adipocytes rather than whole adipose tissue. Whilst this approach has given novel data about the ECS in adipocytes and increased knowledge in this area, in hindsight, it would have been informative to include the same FAAH and MGL activity investigations in the stromal-vascular fraction of adipose tissue. This would have allowed the activities of FAAH and MGL in mature adipocytes to be directly compared to the level of activity in adipose tissue as a whole. This is an important consideration for future studies, as it has been shown that CB₁ mRNA levels are higher in stromal-vascular cells of adipose tissue than mature adipocytes (Bennetzen *et al.*, 2010), but this has not been investigated for FAAH or MGL. Detailed information on the distribution of ECS activity in different cells in adipose tissue may also be of use when comparing samples from lean and obese subjects, as the composition of adipose tissue varies between these groups (Weisberg *et al.*, 2003; Arner *et al.*, 2010).

The results of Chapter 4 showed that FAAH activity in subcutaneous mature adipocytes increased with BMI. It might therefore be expected that the FAAH activities in subcutaneous adipocytes from the obese patients used in Chapter 5 would be higher than the activities seen in Chapter 4. However, the results from the two chapters cannot be directly compared, as the adipose tissue samples were obtained differently. The samples from healthy volunteers were taken with a needle under local anaesthetic, whereas the samples from surgical patients were dissected out with a scalpel under general anaesthetic towards the end of surgery. It has been shown that propofol, a common drug used in general anaesthesia, inhibits FAAH activity (Patel *et al.*, 2003), so the samples cannot be directly compared. In light of this, a future study could be designed to recruit volunteers both with and without metabolic disorders and take needle biopsies, such as in Chapter 4. One of the other benefits of this would be the potential recruitment of diabetic patients without tight glycaemic regulation, as the surgical patients were under extensive primary care prior to surgery.

It is important to note the limitations of experiments performed using cell cultures. Whilst the studies reported in Chapters 6 and 7 of this thesis have value in trying to elucidate simple mechanisms involved in endocannabinoid signalling, the cell culture environment cannot be considered to be comparable to the *in vivo* environment of adipose tissue. The results of Chapter 6 showed that adipokine secretion is not stimulated by endocannabinoids, but it is possible that *in vivo* other factors may promote the endocannabinoids to have effects that are not seen *in vitro*. This may be an important consideration, as the "entourage" effect, in which the signalling of ligands is affected by other structurally related molecules, has been reported repeatedly in endocannabinoid pharmacology (for examples see Smart *et al.*, 2002; Ho *et al.*, 2008; Garcia Mdel *et al.*, 2009).

In Chapter 7, FAAH activity was not found in the cultured differentiated adipocytes. This is very important finding, considering the high levels of FAAH activity seen in adipocytes isolated from healthy, obese and diabetic rats and humans (Chapters 3, 4 and 5). This strongly indicates that at least some components of the ECS are regulated by factors that are not present in the cell culture media, or that available adipocyte cell cultures do not have normal expression of all the components of the ECS, and emphasises the need to interpret cell culture results with caution. A key area that could be addressed in future studies would be to identify physiological factors which induce FAAH activity in cultured adipocytes. This would both provide data on the potential *in vivo* regulation of the ECS, and provide a better cell culture model for investigating the ECS.

The activities of the catabolic enzymes reported in this thesis give an accurate estimate of the rate of endocannabinoid degradation between samples, but in future studies more could be done to investigate the other mechanisms by which endocannabinoid levels are regulated. In particular, measuring the activities of NAPE-PLD and DAGL, the major enzymes required for anandamide and 2-AG synthesis. In conjunction with endocannabinoid concentrations in the samples, this would give a more detailed picture of endocannabinoid regulation and the effects of obesity and diabetes.

8.2 Conclusion

The overarching aim of this thesis was to investigate the role of the ECS in adipocytes in health, obesity and metabolic dysfunction. It has been demonstrated, for the first time, that the rate of anandamide degradation is increased in obesity, whereas 2-AG catabolism by MGL is unaltered in obesity and metabolic dysfunction in humans. Insulin and glucose appear not to affect endocannabinoid degradation rates *in vivo*, but *in vitro* insulin increases anandamide uptake, and insulin and glucose suppress MGL activity. Adipocyte culture work showed that the endocannabinoids do not appear to affect adipokine secretion, and are therefore unlikely to affect adiposity through this mechanism. This study supports the notion of dysregulation of the endocannabinoid system in obesity, and overall the results indicate that this is driven by total or visceral adiposity rather than any of the specific co-morbidity markers associated with obesity.

References

- ALBERTI, K., ECKEL, R., GRUNDY, S., ZIMMET, P., CLEEMAN, J., DONATO, K., FRUCHART, J., JAMES, W., LORIA, C. & SMITH, S. (2009). Harmonizing the Metabolic Syndrome: A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation*, 120, 1640-1645.
- ALEXANDER, S. P. & KENDALL, D. A. (2007). The complications of promiscuity: endocannabinoid action and metabolism. *Br J Pharmacol*, 152, 602-23.
- ALGER, B. E. & KIM, J. (2011). Supply and demand for endocannabinoids. *Trends Neurosci*, 34, 304-15.
- AMERICAN-DIABETES-ASSOCIATION (1997). Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care*, 20, 1183-97.
- ANDERLOVA, K., DOLEZALOVA, R., HOUSOVA, J., BOSANSKA, L., HALUZIKOVA, D., KREMEN, J., SKRHA, J. & HALUZIK, M. (2007). Influence of PPAR-alpha agonist fenofibrate on insulin sensitivity and selected adipose tissue-derived hormones in obese women with type 2 diabetes. *Physiol Res*, 56, 579-86.
- ANDRE, A. & GONTHIER, M. P. (2010). The endocannabinoid system: its roles in energy balance and potential as a target for obesity treatment. *Int J Biochem Cell Biol*, 42, 1788-801.
- ANNUZZI, G., PISCITELLI, F., DI MARINO, L., PATTI, L., GIACCO, R., COSTABILE, G., BOZZETTO, L., RICCARDI, G., VERDE, R., PETROSINO, S., RIVELLESE, A. A. & DI MARZO, V. (2010). Differential alterations of the concentrations of endocannabinoids and related lipids in the subcutaneous adipose tissue of obese diabetic patients. *Lipids Health Dis*, 9, 43.
- ARITA, Y., KIHARA, S., OUCHI, N., TAKAHASHI, M., MAEDA, K., MIYAGAWA, J., HOTTA, K., SHIMOMURA, I., NAKAMURA, T., MIYAOKA, K., KURIYAMA, H., NISHIDA, M., YAMASHITA, S., OKUBO, K., MATSUBARA, K., MURAGUCHI, M., OHMOTO, Y., FUNAHASHI, T. & MATSUZAWA, Y. (1999). Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun*, 257, 79-83.
- ARNER, E., WESTERMARK, P. O., SPALDING, K. L., BRITTON, T., RYDEN, M., FRISEN, J., BERNARD, S. & ARNER, P. (2010). Adipocyte turnover: relevance to human adipose tissue morphology. *Diabetes*, 59, 105-9.
- ARNER, P. (1988). Control of lipolysis and its relevance to development of obesity in man. *Diabetes Metab Rev*, 4, 507-15.
- ARONNE, L. J., TONSTAD, S., MORENO, M., GANTZ, I., ERONDU, N., SURYAWANSHI, S., MOLONY, C., SIEBERTS, S., NAYEE, J., MEEHAN, A. G., SHAPIRO, D., HEYMSFIELD, S. B., KAUFMAN, K. D. & AMATRUDA, J. M. (2010). A clinical trial assessing the safety and efficacy of taranabant, a

CB1R inverse agonist, in obese and overweight patients: a high-dose study. *Int J Obes (Lond)*, 34, 919-35.

ASTARITA, G., AHMED, F. & PIOMELLI, D. (2008). Identification of biosynthetic precursors for the endocannabinoid anandamide in the rat brain. *J Lipid Res*, 49, 48-57.

AUBOEUF, D., RIEUSSET, J., FAJAS, L., VALLIER, P., FRERING, V., RIOU, J. P., STAELS, B., AUWERX, J., LAVILLE, M. & VIDAL, H. (1997). Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator-activated receptors and liver X receptor- α in humans: no alteration in adipose tissue of obese and NIDDM patients. *Diabetes*, 46, 1319-27.

AUORE, A. & GONTHIER, M.-P. (2010). The endocannabinoid system: Its roles in energy balance and potential as a target for obesity treatment *Int J Biochem Cell Biol*, 42, 1788-1801.

BAKKER, A. H., NIJHUIS, J., BUURMAN, W. A., VAN DIELEN, F. M. & GREVE, J. W. (2006). Low number of omental preadipocytes with high leptin and low adiponectin secretion is associated with high fasting plasma glucose levels in obese subjects. *Diabetes Obes Metab*, 8, 585-8.

BANKS, W. A., COON, A. B., ROBINSON, S. M., MOINUDDIN, A., SHULTZ, J. M., NAKAOKE, R. & MORLEY, J. E. (2004). Triglycerides induce leptin resistance at the blood-brain barrier. *Diabetes*, 53, 1253-60.

BARANOVA, A., GOWDER, S. J., SCHLAUCH, K., ELARINY, H., COLLANTES, R., AFENDY, A., ONG, J. P., GOODMAN, Z., CHANDHOKE, V. & YOUNOSS, Z. M. (2006). Gene expression of leptin, resistin, and adiponectin in the white adipose tissue of obese patients with non-alcoholic fatty liver disease and insulin resistance. *Obes Surg*, 16, 1118-25.

BARANOWSKA, B., BARANOWSKA-BIK, A., BIK, W. & MARTYNSKA, L. (2008). The role of leptin and orexins in the dysfunction of hypothalamo-pituitary-gonadal regulation and in the mechanism of hyperactivity in patients with anorexia nervosa. *Neuro Endocrinol Lett*, 29, 37-40.

BATETTA, B., GRIINARI, M., CARTA, G., MURRU, E., LIGRESTI, A., CORDEDDU, L., GIORDANO, E., SANNA, F., BISOGNO, T., UDA, S., COLLU, M., BRUHEIM, I., DI MARZO, V. & BANNI, S. (2009). Endocannabinoids may mediate the ability of (n-3) fatty acids to reduce ectopic fat and inflammatory mediators in obese Zucker rats. *J Nutr*, 139, 1495-501.

BEGG, M., MO, F. M., OFFERTALER, L., BATKAI, S., PACHER, P., RAZDAN, R. K., LOVINGER, D. M. & KUNOS, G. (2003). G protein-coupled endothelial receptor for atypical cannabinoid ligands modulates a Ca^{2+} -dependent K^{+} current. *J Biol Chem*, 278, 46188-94.

BELTRAMO, M., STELLA, N., CALIGNANO, A., LIN, S., MAKRIYANNIS, A. & PIOMELLI, D. (1997). Functional Role of High-Affinity Anandamide Transport, as Revealed by Selective Inhibition. *Science*, 277, 1094-1097.

BENNETZEN, M. F., NIELSEN, T. S., PAULSEN, S. K., BENDIX, J., FISKE, S., JESSEN, N., LUND, S., RICHELSEN, B. & PEDERSEN, S. B. (2010). Reduced cannabinoid receptor 1 protein in subcutaneous adipose tissue of obese. *Eur J Clin Invest*, 40, 121-6.

BENNETZEN, M. F., WELLNER, N., AHMED, S. S., AHMED, S. M., DIEP, T. A., HANSEN, H. S., RICHELSEN, B. & PEDERSEN, S. B. (2011). Investigations of the human endocannabinoid system in two subcutaneous adipose tissue depots in lean subjects and in obese subjects before and after weight loss. *Int J Obes (Lond)*.

BENSAID, M., GARY-BOBO, M., ESCLANGON, A., MAFFRAND, J. P., LE FUR, G., OURY-DONAT, F. & SOUBRIE, P. (2003). The cannabinoid CB1 receptor antagonist SR141716 increases Acrp30 mRNA expression in adipose tissue of obese fa/fa rats and in cultured adipocyte cells. *Mol Pharmacol*, 63, 908-14.

BERMUDEZ-SILVA, F., SUAREZ, J., BAIXERAS, E., COBO, N., BAUTISTA, D., CUESTA-MUNOZ, A., FUENTES, E., JUAN-PICO, P., CASTRO, M., MILMAN, G., MECHOULAM, R., NADAL, A. & RODRIGUEZ DE FONSECA, F. (2008). Presence of functional cannabinoid receptors in human endocrine pancreas. *Diabetologia*, 51, 476-487.

BERMUDEZ-SILVA, F., VIVEROS, M., MCPARTLAND, J. & RODRIGUEZ DE FONSECA, F. (2010). The endocannabinoid system, eating behavior and energy homeostasis: the end or a new beginning? *Pharm Biochem Behav*, 95, 375-382.

BIRO, F. M. & WIEN, M. (2010). Childhood obesity and adult morbidities. *Am J Clin Nutr*, 91, 1499S-1505S.

BISOGNO, T. (2008). Endogenous cannabinoids: structure and metabolism. *J Neuroendocrinol*, 20 Suppl 1, 1-9.

BISOGNO, T., HOWELL, F., WILLIAMS, G., MINASSI, A., CASCIO, M. G., LIGRESTI, A., MATIAS, I., SCHIANO-MORIELLO, A., PAUL, P., WILLIAMS, E. J., GANGADHARAN, U., HOBBS, C., DI MARZO, V. & DOHERTY, P. (2003). Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J Cell Biol*, 163, 463-8.

BISOGNO, T., MELCK, D., BOBROV, M., GRETSKAYA, N. M., BEZUGLOV, V. V., DE PETROCELLIS, L. & DI MARZO, V. (2000). N-acyl-dopamines: novel synthetic CB(1) cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity in vitro and in vivo. *Biochem J*, 351 Pt 3, 817-24.

BISOGNO, T., MELCK, D., DE PETROCELLIS, L. & DI MARZO, V. (1999). Phosphatidic acid as the biosynthetic precursor of the endocannabinoid 2-arachidonoylglycerol in intact mouse neuroblastoma cells stimulated with ionomycin. *J Neurochem*, 72, 2113-9.

BLANKMAN, J. L., SIMON, G. M. & CRAVATT, B. F. (2007). A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. *Chem Biol*, 14, 1347-56.

BLÜHER, M., ENGELI, S., KLÖTING, N., BERNDT, J., FASSHAUER, M., BÁTKAI, S., PACHER, P., SCHÖN, M., JORDAN, J. & STUMVOLI, M. (2006). Dysregulation of the Peripheral and Adipose Tissue Endocannabinoid System in Human Abdominal Obesity. *Diabetes*, 55, 3053-3060.

BOLDROP, L., WILSON, S., BARBIER, A. & FOWLER, C. (2004). A simple stopped assay for fatty acid amide hydrolase avoiding the use of a chloroform extraction phase. *J Biochem Biophys Meth*, 60, 171-177.

BOLINDER, J., KERCKHOFFS, D., MOBERG, E., HAGSTRÖM-TOFT, E. & ARNER, P. (2000). Rates of Skeletal Muscle and Adipose Tissue Glycerol Release in Nonobese and Obese Subjects. *Diabetes*, 49, 797-802.

BORDICCHIA, M., BATTISTONI, I., MANCINELLI, L., GIANNINI, E., REFI, G., MINARDI, D., MUZZONIGRO, G., MAZZUCCHELLI, R., MONTIRONI, R., PISCITELLI, F., PETROSINO, S., DESSI-FULGHERI, P., RAPPELLI, A., DI MARZO, V. & SARZANI, R. (2009). Cannabinoid CB1 receptor expression in relation to visceral adipose depots, endocannabinoid levels, microvascular damage, and the presence of the Cnr1 A3813G variant in humans. *Metabolism*, 59, 734-41.

BOUABOULA, M., HILAIRET, S., MARCHAND, J., FAJAS, L., LE FUR, G. & CASELLAS, P. (2005). Anandamide induced PPARgamma transcriptional activation and 3T3-L1 preadipocyte differentiation. *Eur J Pharmacol*, 517, 174-81.

BRACEY, M. H., HANSON, M. A., MASUDA, K. R., STEVENS, R. C. & CRAVATT, B. F. (2002). Structural adaptations in a membrane enzyme that terminates endocannabinoid signaling. *Science*, 298, 1793-6.

BRAY, G. A., JABLONSKI, K. A., FUJIMOTO, W. Y., BARRETT-CONNOR, E., HAFFNER, S., HANSON, R. L., HILL, J. O., HUBBARD, V., KRISKA, A., STAMM, E. & PI-SUNYER, F. X. (2008). Relation of central adiposity and body mass index to the development of diabetes in the Diabetes Prevention Program. *Am J Clin Nutr*, 87, 1212-8.

BREIVOGEL, C. S., GRIFFIN, G., DI MARZO, V. & MARTIN, B. R. (2001). Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. *Mol Pharmacol*, 60, 155-63.

BUETTNER, C., MUSE, E., CHENG, A., CHEN, L., SCHERER, T., POCAI, A., SU, K., CHENG, B., LI, X., HARVEY-WHITE, J., SCHWARTZ, G., KUNOS, G. & ROSSETTI, L. (2008). Leptin controls adipose tissue lipogenesis via central, STAT3-independent mechanisms. *Nature Medicine*, 14, 667-675.

BURSTEIN, S. H., ROSSETTI, R. G., YAGEN, B. & ZURIER, R. B. (2000). Oxidative metabolism of anandamide. *Prostaglandins Other Lipid Mediat*, 61, 29-41.

CABRAL, G. A., RABORN, E. S., GRIFFIN, L., DENNIS, J. & MARCIANO-CABRAL, F. (2008). CB2 receptors in the brain: role in central immune function. *Br J Pharmacol*, 153, 240-51.

CARRIBA, P., ORTIZ, O., PATKAR, K., JUSTINOVA, Z., STROIK, J., THEMANN, A., MULLER, C., WOODS, A. S., HOPE, B. T., CIRUELA, F., CASADO, V., CANELA, E. I., LLUIS, C., GOLDBERG, S. R., MORATALLA, R., FRANCO, R. & FERRE, S. (2007). Striatal adenosine A2A and cannabinoid CB1 receptors form functional heteromeric complexes that mediate the motor effects of cannabinoids. *Neuropsychopharmacology*, 32, 2249-59.

CAVUOTO, P., MCAINCH, A. J., HATZINIKOLAS, G., CAMERON-SMITH, D. & WITTERT, G. A. (2007a). Effects of cannabinoid receptors on skeletal muscle oxidative pathways. *Mol Cell Endocrinol*, 267, 63-9.

CAVUOTO, P., MCAINCH, A. J., HATZINIKOLAS, G., JANOVSKA, A., GAME, P. & WITTERT, G. A. (2007b). The expression of receptors for endocannabinoids in human and rodent skeletal muscle. *Biochem Biophys Res Commun*, 364, 105-10.

CHANDA, P., GAO, Y., MARK, L., BTESH, J., STRASSLE, B., LU, P., PIESLA, M., ZHANG, M., BINGHAM, B., UVEGES, A., KOWAL, D., GARBE, D., KOURANOVA, E., RING, R., BATES, B., PANGALOS, M., KENNEDY, J., WHITESIDE, G. & SAMAD, T. (2010). Monoacylglycerol Lipase Activity Is a Critical Modulator of the Tone and Integrity of the Endocannabinoid System. *Mol Pharmacol*, 78, 996-1003.

CHEN, L., JONES, P. & PERSAUD, S. (2010). Cannabinoid Receptors are Coupled to Stimulation of Insulin Secretion from Mouse MIN6 beta -cells. *Cell Phys Biochem*, 26, 187-196.

CHEN, M. P., CHUNG, F. M., CHANG, D. M., TSAI, J. C., HUANG, H. F., SHIN, S. J. & LEE, Y. J. (2006). Elevated plasma level of visfatin/pre-B cell colony-enhancing factor in patients with type 2 diabetes mellitus. *J Clin Endocrinol Metab*, 91, 295-9.

CHEN, R. Z., FRASSETTO, A., LAO, J. Z., HUANG, R. R., XIAO, J. C., CLEMENTS, M. J., WALSH, T. F., HALE, J. J., WANG, J., TONG, X. & FONG, T. M. (2008). Pharmacological evaluation of LH-21, a newly discovered molecule that binds to cannabinoid CB1 receptor. *Eur J Pharmacol*, 584, 338-42.

CHRISTENSEN, R., KRISTENSEN, P., BARTELS, E., BLIDDAL, H. & ASTRUP, A. (2007). Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomised trials. *The Lancet*, 370, 1706-1713.

CHRISTOPOULOS, A. & WILSON, K. (2001). Interaction of anandamide with the M(1) and M(4) muscarinic acetylcholine receptors. *Brain Res*, 915, 70-8.

CHU, Z. L., CARROLL, C., CHEN, R., ALFONSO, J., GUTIERREZ, V., HE, H., LUCMAN, A., XING, C., SEBRING, K., ZHOU, J., WAGNER, B., UNETT, D., JONES, R. M., BEHAN, D. P. & LEONARD, J. (2010). N-oleoyldopamine enhances glucose homeostasis through the activation of GPR119. *Mol Endocrinol*, 24, 161-70.

CINAR, R. & SZUCS, M. (2009). CB1 receptor-independent actions of SR141716 on G-protein signaling: coapplication with the mu-opioid agonist Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol unmasks novel, pertussis toxin-insensitive opioid signaling in mu-opioid receptor-Chinese hamster ovary cells. *J Pharmacol Exp Ther*, 330, 567-74.

CONG, L., CHEN, K., LI, J., GAO, P., LI, Q., MI, S., WU, X. & ZHAO, A. Z. (2007). Regulation of adiponectin and leptin secretion and expression by insulin through a PI3K-PDE3B dependent mechanism in rat primary adipocytes. *Biochem J*, 403, 519-25.

CONSIDINE, R. V., NYCE, M. R., KOLACZYNSKI, J. W., ZHANG, P. L., OHANNESIAN, J. P., MOORE, J. H., JR., FOX, J. W. & CARO, J. F. (1997). Dexamethasone stimulates leptin release from human adipocytes: unexpected inhibition by insulin. *J Cell Biochem*, 65, 254-8.

CONSIDINE, R. V., SINHA, M. K., HEIMAN, M. L., KRIAUCIUNAS, A., STEPHENS, T. W., NYCE, M. R., OHANNESIAN, J. P., MARCO, C. C., MCKEE, L. J., BAUER, T. L. & ET AL. (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med*, 334, 292-5.

COTA, D., MARSICANO, G., TSCHOP, M., GRUBLER, Y., FLACHSKAMM, C., SCHUBERT, M., AUER, D., YASSOURIDIS, A., THONE-REINEKE, C., ORTMANN, S., TOMASSONI, F., CERVINO, C., NISOLI, E., LINTHORST, A. C., PASQUALI, R., LUTZ, B., STALLA, G. K. & PAGOTTO, U. (2003). The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J Clin Invest*, 112, 423-31.

COTA, D., SANDOVAL, D., OLIVIERI, M., PRODI, E., D'ALESSIO, D., WOODS, S., SEELEY, R. & OBICI, S. (2009). Food Intake-independent Effects of CB1 Antagonism on Glucose and Lipid Metabolism. *Obesity*.

COTE, M., MATIAS, I., LEMIEUX, I., PETROSINO, S., ALMERAS, N., DESPRES, J. P. & DI MARZO, V. (2007). Circulating endocannabinoid levels, abdominal adiposity and related cardiometabolic risk factors in obese men. *Int J Obes (Lond)*, 31, 692-9.

CRAVATT, B., GIANG, D., MAYFIELD, S., BOGER, D., LERNER, R. & GILULA, N. (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature*, 384, 83-87.

CRAVATT, B. F., DEMAREST, K., PATRICELLI, M. P., BRACEY, M. H., GIANG, D. K., MARTIN, B. R. & LICHTMAN, A. H. (2001). Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci U S A*, 98, 9371-6.

CRESPILO, A., SUAREZ, J., BERMUDEZ-SILVA, F. J., RIVERA, P., VIDA, M., ALONSO, M., PALOMINO, A., LUCENA, M. A., SERRANO, A., PEREZ-MARTIN, M., MACIAS, M., FERNANDEZ-LLEBREZ, P. & RODRIGUEZ DE FONSECA, F. (2010). Expression of the cannabinoid system in muscle: effects of a high-fat diet and CB1 receptor blockade. *Biochem J*, 433, 175-85.

D'EON, T., PIERCE, K., ROIX, J., TYLER, A., CHEN, H. & TEIXEIRA, S. (2008). The Role of Adipocyte Insulin Resistance in the Pathogenesis of Obesity-Related Elevations in Endocannabinoids. *Diabetes*, 57, 1262-1268.

DE GOTTARDI, A., SPAHR, L., RAVIER-DALL'ANTONIA, F. & HADENGUE, A. (2010). Cannabinoid receptor 1 and 2 agonists increase lipid accumulation in hepatocytes. *Liver Int*, 30, 1482-9.

DE LUIS, D., SAGRADO, M., ALLER, R., IZAOLA, O., CONDE, R. & ROMERO, E. (2010a). C358A missense polymorphism of the endocannabinoid degrading enzyme fatty acid amide hydrolase (FAAH) and insulin resistance in patients with diabetes mellitus type 2. *Diab Res Clin Pract*, 88, 76-80.

DE LUIS, D. A., SAGRADO, M. G., ALLER, R., IZAOLA, O. & CONDE, R. (2010b). Effects of C358A missense polymorphism of the degrading enzyme fatty acid amide hydrolase on weight loss, adipocytokines, and insulin resistance after 2 hypocaloric diets. *Metabolism*, 59, 1387-92.

DE PETROCELLIS, L. & DI MARZO, V. (2009). An introduction to the endocannabinoid system: from the early to the latest concepts. *Best Pract Res Clin Endocrinol Metab*, 23, 1-15.

DE PETROCELLIS, L. & DI MARZO, V. (2010). Non-CB1, non-CB2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: focus on G-protein-coupled receptors and transient receptor potential channels. *J Neuroimmune Pharmacol*, 5, 103-21.

DEGAWA-YAMAUCHI, M., BOVENKERK, J. E., JULIAR, B. E., WATSON, W., KERR, K., JONES, R., ZHU, Q. & CONSIDINE, R. V. (2003). Serum resistin (FIZZ3) protein is increased in obese humans. *J Clin Endocrinol Metab*, 88, 5452-5.

DESPRÉS, J.-P. & LEMIEUX, I. (2006). Abdominal obesity and metabolic syndrome. *Nature*, 444, 881-887.

DESPRÉS, J., LEMIEUX, I., BERGERON, J., PIBAROT, P., MATHIEU, P., LAROSE, E., RODÉS-CABAU, J., BERTRAND, O. & POIRIER, P. (2008). Abdominal obesity and the metabolic syndrome: contribution to global cardiometabolic risk. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 28, 1039-1049.

DESPRES, J. P., GOLAY, A. & SJOSTROM, L. (2005). Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. *N Engl J Med*, 353, 2121-34.

DESPRES, J. P., ROSS, R., BOKA, G., ALMERAS, N. & LEMIEUX, I. (2009). Effect of rimonabant on the high-triglyceride/ low-HDL-cholesterol dyslipidemia, intraabdominal adiposity, and liver fat: the ADAGIO-Lipids trial. *Arterioscler Thromb Vasc Biol*, 29, 416-23.

DEURENBERG, P., WESTSTRATE, J. A. & SEIDELL, J. C. (1991). Body mass index as a measure of body fatness: age- and sex-specific prediction formulas. *Br J Nutr*, 65, 105-14.

DEUTSCH, D. G. & CHIN, S. A. (1993). Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem Pharmacol*, 46, 791-6.

DEVANE, W., HANUS, L., BREUER, A., PERTWEE, R., STEVENSON, L., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. & MECHOULAM, R. (1992). Isolation and Structure of a Brain Constituent That Binds to the Cannabinoid Receptor. *Science*, 258, 1946-1949.

DI MARZO, V. (2008). CB(1) receptor antagonism: biological basis for metabolic effects. *Drug Discov Today*, 13, 1026-41.

DI MARZO, V., BISOGNO, T. & DE PETROCELLIS, L. (2007). Endocannabinoids and related compounds: walking back and forth between plant natural products and animal physiology. *Chem Biol*, 14, 741-56.

DI MARZO, V., BISOGNO, T., SUGIURA, T., MELCK, D. & DE PETROCELLIS, L. (1998). The novel endogenous cannabinoid 2-arachidonoylglycerol is inactivated by neuronal- and basophil-like cells: connections with anandamide. *Biochem J*, 331 (Pt 1), 15-9.

DI MARZO, V., CÔTÉ, M., MATIAS, I., LEMIEUX, I., ARSENAULT, B., CARTIER, A., PISCITELLI, F., PETROSINO, S., ALMÉRAS, N. & DESPRÉS, J.-P. (2008). Changes in plasma endocannabinoid levels in viscerally obese men following a 1 year lifestyle modification programme and waist circumference reduction: associations with changes in metabolic risk factors. *Diabetologia*.

DI MARZO, V. & CRISTINO, L. (2008). Why endocannabinoids are not all alike. *Nat Neurosci*, 11, 124-6.

DI MARZO, V., GOPARAJU, S. K., WANG, L., LIU, J., BATKAI, S., JARAI, Z., FEZZA, F., MIURA, G. I., PALMITER, R. D., SUGIURA, T. & KUNOS, G. (2001). Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature*, 410, 822-5.

DI MARZO, V., LIGRESTI, A., MORERA, E., NALLI, M. & ORTAR, G. (2004). The anandamide membrane transporter. Structure-activity relationships of anandamide and oleoylethanolamine analogs with phenyl rings in the polar head group region. *Bioorg Med Chem*, 12, 5161-9.

DI MARZO, V., VERRIJKEN, A., HAKKARAINEN, A., PETROSINO, S., MERTENS, I., LUNDBOM, N., PISCITELLI, F., WESTERBACKA, J., SOROPAAVONEN, A., MATIAS, I., VAN GAAL, L. & TASKINEN, M. R. (2009). Role of insulin as a negative regulator of plasma endocannabinoid levels in obese and nonobese subjects. *Eur J Endocrinol*, 161, 715-22.

DINH, T. P., CARPENTER, D., LESLIE, F. M., FREUND, T. F., KATONA, I., SENSI, S. L., KATHURIA, S. & PIOMELLI, D. (2002a). Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc Natl Acad Sci U S A*, 99, 10819-24.

DINH, T. P., FREUND, T. F. & PIOMELLI, D. (2002b). A role for monoglyceride lipase in 2-arachidonoylglycerol inactivation. *Chem Phys Lipids*, 121, 149-58.

DINH, T. P., KATHURIA, S. & PIOMELLI, D. (2004). RNA interference suggests a primary role for monoacylglycerol lipase in the degradation of the endocannabinoid 2-arachidonoylglycerol. *Mol Pharmacol*, 66, 1260-4.

DIPATRIZIO, N. V. & SIMANSKY, K. J. (2008). Inhibiting parabrachial fatty acid amide hydrolase activity selectively increases the intake of palatable food via cannabinoid CB1 receptors. *Am J Physiol Regul Integr Comp Physiol*, 295, R1409-14.

DUNCAN, R. E., AHMADIAN, M., JAWORSKI, K., SARKADI-NAGY, E. & SUL, H. S. (2007). Regulation of lipolysis in adipocytes. *Annu Rev Nutr*, 27, 79-101.

DURNIN, J. V., DE BRUIN, H. & FEUNIKES, G. I. (1997). Skinfold thicknesses: is there a need to be very precise in their location? *Br J Nutr*, 77, 3-7.

DURNIN, J. V. & WOMERSLEY, J. (1974). Body fat assessed from total body density and its estimation from skinfold thickness: measurements on 481 men and women aged from 16 to 72 years. *Br J Nutr*, 32, 77-97.

ECKARDT, K., SELL, H., TAUBE, A., KOENEN, M., PLATZBECKER, B., CRAMER, A., HERRIGHS, A., LEHTONEN, M., TENNAGELS, N. & ECKEL, J. (2009). Cannabinoid type 1 receptors in human skeletal muscle cells participate in the negative crosstalk between fat and muscle. *Diabetologia*, 52, 664-674.

ELLIS, J., PEDIANI, J. D., CANALS, M., MILASTA, S. & MILLIGAN, G. (2006). Orexin-1 receptor-cannabinoid CB1 receptor heterodimerization results in both ligand-dependent and -independent coordinated alterations of receptor localization and function. *J Biol Chem*, 281, 38812-24.

ELSOHLY, M. A. & SLADE, D. (2005). Chemical constituents of marijuana: the complex mixture of natural cannabinoids. *Life Sci*, 78, 539-48.

ENGELI, S., BÖHNKE, J., FELDPAUSCH, M., GORZELNIAK, K., JANKE, J., BÁTKAL, S., PACHER, P., HARVEY-WHITE, J., LUFT, F., SHARMA, A. & JORDAN, J. (2005). Activation of the Peripheral Endocannabinoid System in Human Obesity. *Diabetes*, 54, 2838-2834.

ENGELI, S., HEUSSER, K., JANKE, J., GORZELNIAK, K., BATKAI, S., PACHER, P., HARVEY-WHITE, J., LUFT, F. C. & JORDAN, J. (2008). Peripheral endocannabinoid system activity in patients treated with sibutramine. *Obesity* 16, 1135-7.

FAIN, J. N., CHEEMA, P. S., BAHOUTH, S. W. & LLOYD HILER, M. (2003). Resistin release by human adipose tissue explants in primary culture. *Biochem Biophys Res Commun*, 300, 674-8.

FOWLER, C. (2007). The contribution of cyclooxygenase-2 to endocannabinoid metabolism and action. *British Journal of Pharmacology*, 152, 594-601.

FOWLER, C. & GHAFOURI, N. (2008). Does the hydrolysis of 2-arachidonoylglycerol regulate its cellular uptake? *Pharm Res*, 58, 72-76.

FOWLER, C. J. (2006). The cannabinoid system and its pharmacological manipulation--a review, with emphasis upon the uptake and hydrolysis of anandamide. *Fundam Clin Pharmacol*, 20, 549-62.

FOWLER, C. J., TIGER, G., LIGRESTI, A., LOPEZ-RODRIGUEZ, M. L. & DI MARZO, V. (2004). Selective inhibition of anandamide cellular uptake versus enzymatic hydrolysis--a difficult issue to handle. *Eur J Pharmacol*, 492, 1-11.

FOWLER, J., JOHNSON, N., HAROLDSON, T., BRINTNALL, J., HERRERA, J., KATZ, S. & BERNLOHR, D. (2009). Regulated renin release from 3T3-L1 adipocytes. *American Journal of Physiology - Endocrinology and Metabolism*, 296, E1383-E1391.

FU, J., GAETANI, S., OVEISI, F., LO VERME, J., SERRANO, A., RODRIGUEZ DE FONSECA, F., ROSENGARTH, A., LUECKE, H., DI GIACOMO, B., TARZIA, G. & PIOMELLI, D. (2003). Oleyethanolamide regulates feeding and body

weight through activation of the nuclear receptor PPAR- α . *Nature*, 425, 90-3.

GALLANT, M., ODEI-ADDO, F., FROST, C. & LEVENDAL, R.-A. (2009). Biological effects of THC and a lipophilic cannabis extract on normal and insulin resistant 3T3-L1 adipocytes. *Phytomedicine*.

GAONI, Y. & MECHOULAM, R. (1964). Isolation Structure and Partial Synthesis of Active Constituent of Hashish. *J Am Chem Soc*, 86, 1646.

GARCIA MDEL, C., ADLER-GRASCHINSKY, E. & CELUCH, S. M. (2009). Enhancement of the hypotensive effects of intrathecally injected endocannabinoids by the entourage compound palmitoylethanolamide. *Eur J Pharmacol*, 610, 75-80.

GARY-BOBO, M., ELACHOURI, G., SCATTON, B., LE FUR, G., OURY-DONAT, F. & BENSAID, M. (2006). The cannabinoid CB1 receptor antagonist rimonabant (SR141716) inhibits cell proliferation and increases markers of adipocyte maturation in cultured mouse 3T3 F442A preadipocytes. *Mol Pharmacol*, 69, 471-8.

GASPERI, V., FEZZA, F., PASQUARIELLO, N., BARI, M., ODDI, S., AGRO, A. F. & MACCARRONE, M. (2007). Endocannabinoids in adipocytes during differentiation and their role in glucose uptake. *Cell Mol Life Sci*, 64, 219-29.

GAVRILA, A., PENG, C. K., CHAN, J. L., MIETUS, J. E., GOLDBERGER, A. L. & MANTZOROS, C. S. (2003). Diurnal and ultradian dynamics of serum adiponectin in healthy men: comparison with leptin, circulating soluble leptin receptor, and cortisol patterns. *J Clin Endocrinol Metab*, 88, 2838-43.

GHAFOURI, N., TIGER, G., RAZDAN, R. K., MAHADEVAN, A., PERTWEE, R. G., MARTIN, B. R. & FOWLER, C. J. (2004). Inhibition of monoacylglycerol lipase and fatty acid amide hydrolase by analogues of 2-arachidonoylglycerol. *Br J Pharmacol*, 143, 774-84.

GLASER, S., ABUMRAD, N., FATADE, F., KACZOCHA, M., STUDHOLME, K. & DEUTSCH, D. (2003). Evidence against the presence of an anandamide transporter. *Proceedings of the National Academy of Sciences*, 100, 4269-4274.

GLASS, C. K. & OGAWA, S. (2006). Combinatorial roles of nuclear receptors in inflammation and immunity. *Nat Rev Immunol*, 6, 44-55.

GONTHIER, M., HOAREAU, L., FESTY, F., MATIAS, I., VALENTI, M., BÈS-HOUTMANN, S., ROUCH, C., ROBERT-DA SILVA, C., CHESNE, S., LEFEBVRE D'HELLEN COURT, C., CÉSARI, M., DI MARZO, V. & ROCHE, R. (2007). Identification of Endocannabinoids and Related Compounds in Human Fat Cells. *Obesity*, 15, 837-845.

GRUNDY, S. M., BREWER, H. B., JR., CLEEMAN, J. I., SMITH, S. C., JR. & LENFANT, C. (2004). Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Arterioscler Thromb Vasc Biol*, 24, e13-8.

GUO, K., MCMINN, J. E., LUDWIG, T., YU, Y. H., YANG, G., CHEN, L., LOH, D., LI, C., CHUA, S., JR. & ZHANG, Y. (2007). Disruption of peripheral leptin signaling in mice results in hyperleptinemia without associated metabolic abnormalities. *Endocrinology*, 148, 3987-97.

HALBERG, N., SCHRAW, T. D., WANG, Z. V., KIM, J. Y., YI, J., HAMILTON, M. P., LUBY-PHELPS, K. & SCHERER, P. E. (2009). Systemic fate of the adipocyte-derived factor adiponectin. *Diabetes*, 58, 1961-70.

HANUS, L., ABU-LAFI, S., FRIDE, E., BREUER, A., VOGEL, Z., SHALEV, D. E., KUSTANOVICH, I. & MECHOULAM, R. (2001). 2-arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor. *Proc Natl Acad Sci U S A*, 98, 3662-5.

HARISMENDY, O., BANSAL, V., BHATIA, G., NAKANO, M., SCOTT, M., WANG, X., DIB, C., TURLOTTE, E., SIPE, J., MURRAY, S., DELEUZE, J., BAFNA, V., TOPOL, E. & FRAZER, K. (2010). Population sequencing of two endocannabinoid metabolic genes identifies rare and common regulatory variants associated with extreme obesity and metabolite level. *Gen Biol*, 11, R118.

HARRISON, C. & TRAYNOR, J. R. (2003). The [35S]GTPgammaS binding assay: approaches and applications in pharmacology. *Life Sci*, 74, 489-508.

HAUGEN, F., JORGENSEN, A., DREVON, C. A. & TRAYHURN, P. (2001). Inhibition by insulin of resistin gene expression in 3T3-L1 adipocytes. *FEBS Lett*, 507, 105-8.

HEILBRONN, L. K., ROOD, J., JANDEROVA, L., ALBU, J. B., KELLEY, D. E., RAVUSSIN, E. & SMITH, S. R. (2004). Relationship between serum resistin concentrations and insulin resistance in nonobese, obese, and obese diabetic subjects. *J Clin Endocrinol Metab*, 89, 1844-8.

HENSTRIDGE, C. M., BALENGA, N. A., FORD, L. A., ROSS, R. A., WALDHOER, M. & IRVING, A. J. (2009). The GPR55 ligand L-alpha-lysophosphatidylinositol promotes RhoA-dependent Ca²⁺ signaling and NFAT activation. *FASEB J*, 23, 183-93.

HERMANN, A., KACZOCHA, M. & DEUTSCH, D. G. (2006). 2-Arachidonoylglycerol (2-AG) membrane transport: history and outlook. *AAPS J*, 8, E409-12.

HERMANN, H., DE PETROCELLIS, L., BISOGNO, T., SCHIANO MORIELLO, A., LUTZ, B. & DI MARZO, V. (2003). Dual effect of cannabinoid CB1 receptor stimulation on a vanilloid VR1 receptor-mediated response. *Cell Mol Life Sci*, 60, 607-16.

HEYMSFIELD, S. B., GREENBERG, A. S., FUJIOKA, K., DIXON, R. M., KUSHNER, R., HUNT, T., LUBINA, J. A., PATANE, J., SELF, B., HUNT, P. & MCCAMISH, M. (1999). Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. *JAMA*, 282, 1568-75.

HILLARD, C. J., EDGEMOND, W. S., JARRAHIAN, A. & CAMPBELL, W. B. (1997). Accumulation of N-arachidonylethanolamine (anandamide) into

cerebellar granule cells occurs via facilitated diffusion. *J Neurochem*, 69, 631-8.

HO, S. Y., DELGADO, L. & STORCH, J. (2002). Monoacylglycerol metabolism in human intestinal Caco-2 cells: evidence for metabolic compartmentation and hydrolysis. *J Biol Chem*, 277, 1816-23.

HO, W. S., BARRETT, D. A. & RANDALL, M. D. (2008). 'Entourage' effects of N-palmitoylethanolamide and N-oleoylethanolamide on vasorelaxation to anandamide occur through TRPV1 receptors. *Br J Pharmacol*, 155, 837-46.

HO, W. S. & HILLARD, C. J. (2005). Modulators of endocannabinoid enzymic hydrolysis and membrane transport. *Handb Exp Pharmacol*, 187-207.

HOCHBERG, I. & HOCHBERG, Z. (2010). Expanding the definition of hypothalamic obesity. *Obes Rev*, 11, 709-21.

HOLLANDER, P. A., AMOD, A., LITWAK, L. E. & CHAUDHARI, U. (2010). Effect of rimonabant on glycemic control in insulin-treated type 2 diabetes: the ARPEGGIO trial. *Diabetes Care*, 33, 605-7.

HOSOHATA, K., QUOCK, R. M., HOSOHATA, Y., BURKEY, T. H., MAKRIYANNIS, A., CONSROE, P., ROESKE, W. R. & YAMAMURA, H. I. (1997). AM630 is a competitive cannabinoid receptor antagonist in the guinea pig brain. *Life Sci*, 61, PL115-8.

HOTTA, K., FUNAHASHI, T., ARITA, Y., TAKAHASHI, M., MATSUDA, M., OKAMOTO, Y., IWAHASHI, H., KURIYAMA, H., OUCHI, N., MAEDA, K., NISHIDA, M., KIHARA, S., SAKAI, N., NAKAJIMA, T., HASEGAWA, K., MURAGUCHI, M., OHMOTO, Y., NAKAMURA, T., YAMASHITA, S., HANAFUSA, T. & MATSUZAWA, Y. (2000). Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol*, 20, 1595-9.

IZZO, A., PISCITELLI, F., CAPASSO, R., AVIELLO, G., ROMANO, B., BORRELLI, F., PETROSINO, S. & DI MARZO, V. (2009). Peripheral endocannabinoid dysregulation in obesity: relation to intestinal motility and energy processing induced by food deprivation and re-feeding. *Br J Pharmacol*, 0007-1188/09.

JAMSHIDI, N. & TAYLOR, D. A. (2001). Anandamide administration into the ventromedial hypothalamus stimulates appetite in rats. *Br J Pharmacol*, 134, 1151-4.

JANKE, J., ENGELI, S., GORZELNIAK, K., LUFT, F. C. & SHARMA, A. M. (2002). Resistin gene expression in human adipocytes is not related to insulin resistance. *Obes Res*, 10, 1-5.

JARAI, Z., WAGNER, J. A., VARGA, K., LAKE, K. D., COMPTON, D. R., MARTIN, B. R., ZIMMER, A. M., BONNER, T. I., BUCKLEY, N. E., MEZEY, E., RAZDAN, R. K., ZIMMER, A. & KUNOS, G. (1999). Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc Natl Acad Sci U S A*, 96, 14136-41.

JBILLO, O., RAVINET-TRILLOU, C., ARNONE, M., BUISSON, I., BRIBES, E., PELERAUX, A., PENARIER, G., SOUBRIE, P., LE FUR, G., GALIEGUE, S. &

CASELLAS, P. (2005). The CB1 receptor antagonist rimonabant reverses the diet-induced obesity phenotype through the regulation of lipolysis and energy balance. *FASEB J*, 19, 1567-9.

JENKINS, C. M., MANCUSO, D. J., YAN, W., SIMS, H. F., GIBSON, B. & GROSS, R. W. (2004). Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. *J Biol Chem*, 279, 48968-75.

JIANG, H., HE, J., PU, S., TANG, C. & XU, G. (2007). Heat shock protein 70 is translocated to lipid droplets in rat adipocytes upon heat stimulation. *Biochim Biophys Acta*, 1771, 66-74.

KACZOCHA, M., GLASER, S. & DEUTSCH, D. (2009). Identification of intracellular carriers for the endocannabinoid anandamide. *Proc Natl Acad Sci U S A*, 106, 6375-6380.

KACZOCHA, M., HERMANN, A., GLASER, S., BOJESSEN, I. & DEUTSCH, D. (2006). Anandamide Uptake Is Consistent with Rate-limited Diffusion and Is Regulated by the Degree of Its Hydrolysis by Fatty Acid Amide Hydrolase. *J Biol Chem*, 281, 9066-9075.

KARALIOTA, S., SIAFAKA-KAPADAI, A., GONTINO, C., PSARRA, K. & MAVRI-VAVAYANNI, M. (2009). Anandamide increases the differentiation of rat adipocytes and causes PPARgamma and CB1 receptor upregulation. *Obesity (Silver Spring)*, 17, 1830-8.

KARLSSON, M., CONTRERAS, J. A., HELLMAN, U., TORNQVIST, H. & HOLM, C. (1997). cDNA cloning, tissue distribution, and identification of the catalytic triad of monoglyceride lipase. Evolutionary relationship to esterases, lysophospholipases, and haloperoxidases. *J Biol Chem*, 272, 27218-23.

KASER, S., KASER, A., SANDHOFER, A., EBENBICHLER, C. F., TILG, H. & PATSCH, J. R. (2003). Resistin messenger-RNA expression is increased by proinflammatory cytokines in vitro. *Biochem Biophys Res Commun*, 309, 286-90.

KEARN, C. S., BLAKE-PALMER, K., DANIEL, E., MACKIE, K. & GLASS, M. (2005). Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors enhances heterodimer formation: a mechanism for receptor cross-talk? *Mol Pharmacol*, 67, 1697-704.

KEMPF, K., HECTOR, J., STRATE, T., SCHWARZLOH, B., ROSE, B., HERDER, C., MARTIN, S. & ALGENSTAEDT, P. (2007). Immune-mediated activation of the endocannabinoid system in visceral adipose tissue in obesity. *Horm Metab Res*, 39, 596-600.

KIELSTEIN, J. T., BECKER, B., GRAF, S., BRABANT, G., HALLER, H. & FLISER, D. (2003). Increased resistin blood levels are not associated with insulin resistance in patients with renal disease. *Am J Kidney Dis*, 42, 62-6.

KIM, K. H., LEE, K., MOON, Y. S. & SUL, H. S. (2001). A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation. *J Biol Chem*, 276, 11252-6.

KIMURA, T., OHTA, T., WATANABE, K., YOSHIMURA, H. & YAMAMOTO, I. (1998). Anandamide, an endogenous cannabinoid receptor ligand, also interacts with 5-hydroxytryptamine (5-HT) receptor. *Biol Pharm Bull*, 21, 224-6.

KIPNES, M. S., HOLLANDER, P., FUJIOKA, K., GANTZ, I., SECK, T., ERONDU, N., SHENTU, Y., LU, K., SURYAWANSHI, S., CHOU, M., JOHNSON-LEVONAS, A. O., HEYMSFIELD, S. B., SHAPIRO, D., KAUFMAN, K. D. & AMATRUDA, J. M. (2010). A one-year study to assess the safety and efficacy of the CB1R inverse agonist taranabant in overweight and obese patients with type 2 diabetes. *Diabetes Obes Metab*, 12, 517-31.

KIRKHAM, T. C. (2003). Endogenous cannabinoids: a new target in the treatment of obesity. *Am J Physiol Regul Integr Comp Physiol*, 284, R343-4.

KIRKHAM, T. C., WILLIAMS, C. M., FEZZA, F. & DI MARZO, V. (2002). Endocannabinoid levels in rat limbic forebrain and hypothalamus in relation to fasting, feeding and satiation: stimulation of eating by 2-arachidonoyl glycerol. *Br J Pharmacol*, 136, 550-7.

KLEMPEL, M. C. & VARADY, K. A. (2011). Reliability of leptin, but not adiponectin, as a biomarker for diet-induced weight loss in humans. *Nutr Rev*, 69, 145-54.

KONDO, S., KONDO, H., NAKANE, S., KODAKA, T., TOKUMURA, A., WAKU, K. & SUGIURA, T. (1998). 2-Arachidonoylglycerol, an endogenous cannabinoid receptor agonist: identification as one of the major species of monoacylglycerols in various rat tissues, and evidence for its generation through CA2+-dependent and -independent mechanisms. *FEBS Letters*, 429, 152-6.

KOZAK, K. R., GUPTA, R. A., MOODY, J. S., JI, C., BOEGLIN, W. E., DUBOIS, R. N., BRASH, A. R. & MARNETT, L. J. (2002). 15-Lipoxygenase metabolism of 2-arachidonoylglycerol. Generation of a peroxisome proliferator-activated receptor alpha agonist. *J Biol Chem*, 277, 23278-86.

KOZAK, K. R., ROWLINSON, S. W. & MARNETT, L. J. (2000). Oxygenation of the endocannabinoid, 2-arachidonoylglycerol, to glyceryl prostaglandins by cyclooxygenase-2. *J Biol Chem*, 275, 33744-9.

KURAHASHI, Y., UEDA, N., SUZUKI, H., SUZUKI, M. & YAMAMOTO, S. (1997). Reversible hydrolysis and synthesis of anandamide demonstrated by recombinant rat fatty-acid amide hydrolase. *Biochem Biophys Res Commun*, 237, 512-5.

KURZ, C. M., GOTTSCHALK, C., SCHLICKER, E. & KATHMANN, M. (2008). Identification of a presynaptic cannabinoid CB1 receptor in the guinea-pig atrium and sequencing of the guinea-pig CB1 receptor. *J Physiol Pharmacol*, 59, 3-15.

LABAR, G., BAUVOIS, C., BOREL, F., FERRER, J. L., WOUTERS, J. & LAMBERT, D. M. (2010a). Crystal structure of the human monoacylglycerol lipase, a key actor in endocannabinoid signaling. *Chembiochem*, 11, 218-27.

LABAR, G., WOUTERS, J. & LAMBERT, D. M. (2010b). A review on the monoacylglycerol lipase: at the interface between fat and endocannabinoid signalling. *Curr Med Chem*, 17, 2588-607.

LAFONTAN, M. & LANGIN, D. (2009). Lipolysis and lipid mobilization in human adipose tissue. *Prog Lipid Res*, 48, 275-97.

LAMBERT, D. M. & MUCCIOLI, G. G. (2007). Endocannabinoids and related N-acylethanolamines in the control of appetite and energy metabolism: emergence of new molecular players. *Curr Opin Clin Nutr Metab Care*, 10, 735-44.

LATORRE, R., BRAUCHI, S., ORTA, G., ZAEZER, C. & VARGAS, G. (2007). ThermoTRP channels as modular proteins with allosteric gating. *Cell Calcium*, 42, 427-38.

LAUCKNER, J. E., JENSEN, J. B., CHEN, H. Y., LU, H. C., HILLE, B. & MACKIE, K. (2008). GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. *Proc Natl Acad Sci U S A*, 105, 2699-704.

LEE, M. J. & FRIED, S. K. (2009). Integration of hormonal and nutrient signals that regulate leptin synthesis and secretion. *Am J Physiol Endocrinol Metab*, 296, E1230-8.

LENMAN, A. & FOWLER, C. J. (2007). Interaction of ligands for the peroxisome proliferator-activated receptor gamma with the endocannabinoid system. *Br J Pharmacol*, 151, 1343-51.

LEONARD, B., WATSON, R., LOOMES, K., PHILLIPS, A. & COOPER, G. (2005). Insulin resistance in the Zucker diabetic fatty rat: a metabolic characterisation of obese and lean phenotypes. *Acta Diabetologica*, 42, 162-170.

LEUNG, D., SAGHATELIAN, A., SIMON, G. M. & CRAVATT, B. F. (2006). Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. *Biochemistry*, 45, 4720-6.

LICHTMAN, A. H., HAWKINS, E. G., GRIFFIN, G. & CRAVATT, B. F. (2002). Pharmacological activity of fatty acid amides is regulated, but not mediated, by fatty acid amide hydrolase in vivo. *J Pharmacol Exp Ther*, 302, 73-9.

LIGRESTI, A., DE PETROCELLIS, L., HERNAN PEREZ DE LA OSSA, D., ABERTURAS, R., CRISTINO, L., MORIELLO, A. S., FINIZIO, A., GIL, M. E., TORRES, A. I., MOLPECERES, J. & DI MARZO, V. (2010). Exploiting nanotechnologies and TRPV1 channels to investigate the putative anandamide membrane transporter. *PLoS One*, 5, e10239.

LINDBORG, K. A., TEACHEY, M. K., JACOB, S. & HENRIKSEN, E. J. (2010). Effects of in vitro antagonism of endocannabinoid-1 receptors on the glucose transport system in normal and insulin-resistant rat skeletal muscle. *Diabetes Obes Metab*, 12, 722-30.

LIU, F., FAN, H. Q., QIU, J., WANG, B., ZHANG, M., GU, N., ZHANG, C. M., FEI, L., PAN, X. Q., GUO, M., CHEN, R. H. & GUO, X. R. (2008). A paradox:

insulin inhibits expression and secretion of resistin which induces insulin resistance. *World J Gastroenterol*, 14, 95-100.

LIU, J., WANG, L., HARVEY-WHITE, J., OSEI-HYIAMAN, D., RAZDAN, R., GONG, Q., CHAN, A. C., ZHOU, Z., HUANG, B. X., KIM, H. Y. & KUNOS, G. (2006). A biosynthetic pathway for anandamide. *Proc Natl Acad Sci U S A*, 103, 13345-50.

LIU, Y. L., CONNOLEY, I. P., WILSON, C. A. & STOCK, M. J. (2005). Effects of the cannabinoid CB1 receptor antagonist SR141716 on oxygen consumption and soleus muscle glucose uptake in Lep(ob)/Lep(ob) mice. *Int J Obes (Lond)*, 29, 183-7.

LO VERME, J., FU, J., ASTARITA, G., LA RANA, G., RUSSO, R., CALIGNANO, A. & PIOMELLI, D. (2005). The nuclear receptor peroxisome proliferator-activated receptor- α mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol Pharmacol*, 67, 15-9.

LOFGREN, P., SJOLIN, E., WAHLEN, K. & HOFFSTEDT, J. (2007). Human adipose tissue cannabinoid receptor 1 gene expression is not related to fat cell function or adiponectin level. *J Clin Endocrinol Metab*, 92, 1555-9.

LONG, J. Z., NOMURA, D. K. & CRAVATT, B. F. (2009). Characterization of monoacylglycerol lipase inhibition reveals differences in central and peripheral endocannabinoid metabolism. *Chem Biol*, 16, 744-53.

MACCARRONE, M., DAINESE, E. & ODDI, S. (2010). Intracellular trafficking of anandamide: new concepts for signaling. *Trends Biochem Sci*, 35, 601-8.

MACCARRONE, M., DI RIENZO, M., FINAZZI-AGRÒ, A. & ROSSI, A. (2003). Leptin activates the anandamide hydrolase promoter in human T lymphocytes through STAT3. *J Biol Chem*, 278, 13318-24.

MACCARRONE, M., GASPERI, V., FEZZA, F., FINAZZI-AGRÒ, A. & ROSSI, A. (2004). Differential regulation of fatty acid amide hydrolase promoter in human immune cells and neuronal cells by leptin and progesterone. *European Journal of Biochemistry*, 271, 4666-76.

MACKIE, K. (2008). Cannabinoid receptors: where they are and what they do. *J Neuroendocrinol*, 20 Suppl 1, 10-4.

MALLAT, A., TEIXEIRA-CLERC, F., DEVEAUX, V., MANIN, S. & LOTERSZTAJN, S. (2011). The Endocannabinoid System as a Key Mediator during Liver Diseases : New Insights and Therapeutic Openings. *Br J Pharmacol*.

MANCINI, G., QUARTA, C., SRIVASTAVA, R., KLAUS, S., PAGOTTO, U. & LUTZ, B. Year. Adipocyte-specific CB1 conditional knock-out mice: new insights in the study of obesity and metabolic syndrome. In: 20th Annual Symposium on the Cannabinoids, 2010 Lund, Sweden. Research Triangle Park, 17.

MARSH, S., POWELL, P., AGARWAL, A., DELL'ITALIA, L. & CHATHAM, J. (2007). Cardiovascular dysfunction in Zucker obese and Zucker diabetic fatty rats: role of hydronephrosis. *Am J Physiol Endocrinol Metab*, 293, H292-H298.

MARTIN, B. R., BELETSKAYA, I., PATRICK, G., JEFFERSON, R., WINCKLER, R., DEUTSCH, D. G., DI MARZO, V., DASSE, O., MAHADEVAN, A. & RAZDAN, R. K. (2000). Cannabinoid properties of methylfluorophosphonate analogs. *J Pharmacol Exp Ther*, 294, 1209-18.

MATIAS, I. & DI MARZO, V. (2007). Endocannabinoids and the control of energy balance. *Trends Endocrinol Metab*, 18, 27-37.

MATIAS, I., GONTHIER, M., PETROSINO, S., DOCIMO, L., CAPASSO, R., HOAREAU, L., MONTELEONE, P., ROCHE, R., IZZO, A. & DI MARZO, V. (2007). Role and regulation of acylethanolamides in energy balance: focus on adipocytes and beta-cells. *Br J Pharmacol*, 152, 66-690.

MATIAS, I., GONTHIER, M. P., ORLANDO, P., MARTIADIS, V., DE PETROCELLIS, L., CERVINO, C., PETROSINO, S., HOAREAU, L., FESTY, F., PASQUALI, R., ROCHE, R., MAJ, M., PAGOTTO, U., MONTELEONE, P. & DI MARZO, V. (2006). Regulation, function, and dysregulation of endocannabinoids in models of adipose and beta-pancreatic cells and in obesity and hyperglycemia. *J Clin Endocrinol Metab*, 91, 3171-80.

MATSUDA, L., LOLAIT, S., BROWNSTEIN, M., YOUNG, A. & BONNER, T. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*, 346, 561-564.

MATSUMOTO, T., KANO, K., KONDO, D., FUKUDA, N., IRIBE, Y., TANAKA, N., MATSUBARA, Y., SAKUMA, T., SATOMI, A., OTAKI, M., RYU, J. & MUGISHIMA, H. (2008). Mature Adipocyte-Derived Dedifferentiated Fat Cells Exhibit Multilineage Potential. *J Cell Physiol*, 215, 210-222.

MAURELLI, S., BISOGNO, T., DE PETROCELLIS, L., DI LUCCIA, A., MARINO, G. & DI MARZO, V. (1995). Two novel classes of neuroactive fatty acid amides are substrates for mouse neuroblastoma 'anandamide amidohydrolase'. *FEBS Lett*, 377, 82-6.

MCKINNEY, M. K. & CRAVATT, B. F. (2005). Structure and function of fatty acid amide hydrolase. *Annu Rev Biochem*, 74, 411-32.

MCPARTLAND, J. M., NORRIS, R. W. & KILPATRICK, C. W. (2007). Tempo and mode in the endocannabinoid system. *J Mol Evol*, 65, 267-76.

MCTERNAN, P. G., FISHER, F. M., VALSAMAKIS, G., CHETTY, R., HARTE, A., MCTERNAN, C. L., CLARK, P. M., SMITH, S. A., BARNETT, A. H. & KUMAR, S. (2003). Resistin and type 2 diabetes: regulation of resistin expression by insulin and rosiglitazone and the effects of recombinant resistin on lipid and glucose metabolism in human differentiated adipocytes. *J Clin Endocrinol Metab*, 88, 6098-106.

MEAD, J. R., IRVINE, S. A. & RAMJI, D. P. (2002). Lipoprotein lipase: structure, function, regulation, and role in disease. *J Mol Med*, 80, 753-69.

MECHOULAM, R., BEN-SHABAT, S., HANUS, L., LIGUMSKY, M., KAMINSKI, N. E., SCHATZ, A. R., GOPHER, A., ALMOG, S., MARTIN, B. R., COMPTON, D. R. & ET AL. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol*, 50, 83-90.

- MECHOULAM, R., BERRY, E. M., AVRAHAM, Y., DI MARZO, V. & FRIDE, E. (2006). Endocannabinoids, feeding and suckling--from our perspective. *Int J Obes (Lond)*, 30 Suppl 1, S24-8.
- MECHOULAM, R., PETERS, M., MURILLO-RODRIGUEZ, E. & HANUS, L. O. (2007). Cannabidiol--recent advances. *Chem Biodivers*, 4, 1678-92.
- MELIS, T., SUCCU, S., SANNA, F., BOI, A., ARGIOLAS, A. & MELIS, M. R. (2007). The cannabinoid antagonist SR 141716A (Rimonabant) reduces the increase of extra-cellular dopamine release in the rat nucleus accumbens induced by a novel high palatable food. *Neurosci Lett*, 419, 231-5.
- METAIS, C., FORCHERON, F., ABDALLAH, P., BASSET, A., CARMINE, P., BRICCA, G. & BEYLOT, M. (2008). Adiponectin receptors: expression in Zucker diabetic rats and effects of fenofibrate and metformin. *Metabolism Clinical and Experimental*, 57, 946-953.
- MEZEY, E., TOTH, Z. E., CORTRIGHT, D. N., ARZUBI, M. K., KRAUSE, J. E., ELDE, R., GUO, A., BLUMBERG, P. M. & SZALLASI, A. (2000). Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. *Proc Natl Acad Sci U S A*, 97, 3655-60.
- MOODY, J. S., KOZAK, K. R., JI, C. & MARNETT, L. J. (2001). Selective oxygenation of the endocannabinoid 2-arachidonylglycerol by leukocyte-type 12-lipoxygenase. *Biochemistry*, 40, 861-6.
- MOTAGHEDI, R. & MCGRAW, T. (2008). The CB1 Endocannabinoid System Modulates Adipocyte Insulin Sensitivity. *Obesity*, 16, 1727-1734.
- MOTOSHIMA, H., WU, X., SINHA, M. K., HARDY, V. E., ROSATO, E. L., BARBOT, D. J., ROSATO, F. E. & GOLDSTEIN, B. J. (2002). Differential regulation of adiponectin secretion from cultured human omental and subcutaneous adipocytes: effects of insulin and rosiglitazone. *J Clin Endocrinol Metab*, 87, 5662-7.
- MUCCIOLI, G. G. (2010). Endocannabinoid biosynthesis and inactivation, from simple to complex. *Drug Discovery Today*, 15, 474-83.
- MUKHOPADHYAY, S., CHAPNICK, B. M. & HOWLETT, A. C. (2002). Anandamide-induced vasorelaxation in rabbit aortic rings has two components: G protein dependent and independent. *Am J Physiol Heart Circ Physiol*, 282, H2046-54.
- MULLER, T. D., REICHWALD, K., WERMTER, A. K., BRONNER, G., NGUYEN, T. T., FRIEDEL, S., KOBERWITZ, K., ENGEL, S., LICHTNER, P., MEITINGER, T., SCHAFER, H., HEBEBRAND, J. & HINNEY, A. (2007). No evidence for an involvement of variants in the cannabinoid receptor gene (CNR1) in obesity in German children and adolescents. *Mol Genet Metab*, 90, 429-34.
- MUNRO, S., THOMAS, K. & ABU-SHAAR, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature*, 365, 61-65.
- MURDOLO, G., KEMPF, K., HAMMARSTEDT, A., HERDER, C., SMITH, U. & JANSSEN, P. A. (2007). Insulin differentially modulates the peripheral

endocannabinoid system in human subcutaneous abdominal adipose tissue from lean and obese individuals. *J Endocrinol Invest*, 30, RC17-21.

NAGAEV, I. & SMITH, U. (2001). Insulin resistance and type 2 diabetes are not related to resistin expression in human fat cells or skeletal muscle. *Biochem Biophys Res Commun*, 285, 561-4.

NISHIZAWA, H., SHIMOMURA, I., KISHIDA, K., MAEDA, N., KURIYAMA, H., NAGARETANI, H., MATSUDA, M., KONDO, H., FURUYAMA, N., KIHARA, S., NAKAMURA, T., TOCHINO, Y., FUNAHASHI, T. & MATSUZAWA, Y. (2002). Androgens decrease plasma adiponectin, an insulin-sensitizing adipocyte-derived protein. *Diabetes*, 51, 2734-41.

NOBUSUE, H., ENDO, T. & KANO, K. (2008). Establishment of a preadipocyte cell line derived from mature adipocytes of GFP transgenic mice and formation of adipose tissue. *Cell and Tissue Research*, 332, 435-446.

NOGUEIRAS, R., VEYRAT-DUREBEX, C., SUCHANEK, P. M., KLEIN, M., TSCHOP, J., CALDWELL, C., WOODS, S. C., WITTMANN, G., WATANABE, M., LIPOSITS, Z., FEKETE, C., REIZES, O., ROHNER-JEANRENAUD, F. & TSCHOP, M. H. (2008). Peripheral, but not central, CB1 antagonism provides food intake-independent metabolic benefits in diet-induced obese rats. *Diabetes*, 57, 2977-91.

NORTON, K. & OLDS, T. 1996. Measurement Techniques in Anthropometry. In: NORTON, K. & OLDS, T. (eds.) *Anthropometrica*.

O'SULLIVAN, S. E. (2007). Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. *Br J Pharmacol*, 152, 576-82.

O'SULLIVAN, S. E., KENDALL, D. A. & RANDALL, M. D. (2009a). Time-Dependent Vascular Effects of Endocannabinoids Mediated by Peroxisome Proliferator-Activated Receptor Gamma (PPARgamma). *PPAR Res*, 2009, 425289.

O'SULLIVAN, S. E., SUN, Y., BENNETT, A. J., RANDALL, M. D. & KENDALL, D. A. (2009b). Time-dependent vascular actions of cannabidiol in the rat aorta. *Eur J Pharmacol*, 612, 61-8.

O'SULLIVAN, S. E., TARLING, E. J., BENNETT, A. J., KENDALL, D. A. & RANDALL, M. D. (2005). Novel time-dependent vascular actions of Delta9-tetrahydrocannabinol mediated by peroxisome proliferator-activated receptor gamma. *Biochem Biophys Res Commun*, 337, 824-31.

ODDI, S., FEZZA, F., PASQUARIELLO, N., D'AGOSTINO, A., CATANZARO, G., DE SIMONE, C., RAPINO, C., FINAZZI-AGRO, A. & MACCARRONE, M. (2009). Molecular identification of albumin and Hsp70 as cytosolic anandamide-binding proteins. *Chem Biol*, 16, 624-32.

ODDI, S., FEZZA, F., PASQUARIELLO, N., DE SIMONE, C., RAPINO, C., DAINESE, E., FINAZZI-AGRO, A. & MACCARRONE, M. (2008). Evidence for the intracellular accumulation of anandamide in adiposomes. *Cell Mol Life Sci*, 65, 840-50.

OGAWA, Y., MASUZAKI, H., ISSE, N., OKAZAKI, T., MORI, K., SHIGEMOTO, M., SATOH, N., TAMURA, N., HOSODA, K., YOSHIMASA, Y. & ET AL. (1995). Molecular cloning of rat obese cDNA and augmented gene expression in genetically obese Zucker fatty (fa/fa) rats. *J Clin Invest*, 96, 1647-52.

OKAMOTO, Y., MORISHITA, J., TSUBOI, K., TONAI, T. & UEDA, N. (2004). Molecular characterization of a phospholipase D generating anandamide and its congeners. *J Biol Chem*, 279, 5298-305.

ORTEGA-GUTIERREZ, S., HAWKINS, E. G., VISO, A., LOPEZ-RODRIGUEZ, M. L. & CRAVATT, B. F. (2004). Comparison of anandamide transport in FAAH wild-type and knockout neurons: evidence for contributions by both FAAH and the CB1 receptor to anandamide uptake. *Biochemistry*, 43, 8184-90.

OSEI-HYIAMAN, D., DEPETRILLO, M., PACHER, P., LIU, J., RADAIEVA, S., BATKAI, S., HARVEY-WHITE, J., MACKIE, K., OFFERTALER, L., WANG, L. & KUNOS, G. (2005). Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J Clin Invest*, 115, 1298-305.

OSWAL, A. & YEO, G. (2010). Leptin and the control of body weight: a review of its diverse central targets, signaling mechanisms, and role in the pathogenesis of obesity. *Obesity (Silver Spring)*, 18, 221-9.

OVERTON, H. A., BABBS, A. J., DOEL, S. M., FYFE, M. C., GARDNER, L. S., GRIFFIN, G., JACKSON, H. C., PROCTER, M. J., RASAMISON, C. M., TANG-CHRISTENSEN, M., WIDDOWSON, P. S., WILLIAMS, G. M. & REYNET, C. (2006). Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. *Cell Metab*, 3, 167-75.

PAGANO, C., PILON, C., CALCAGNO, A., URBANET, R., ROSSATO, M., MILAN, G., BIANCHI, K., RIZZUTO, R., BERNANTE, P., FEDERSPIL, G. & VETTOR, R. (2007). The endogenous cannabinoid system stimulates glucose uptake in human fat cells via phosphatidylinositol 3-kinase and calcium-dependent mechanisms. *J Clin Endocrinol Metab*, 92, 4810-9.

PAGANO, C., ROSSATO, M. & VETTOR, R. (2008). Endocannabinoids, adipose tissue and lipid metabolism. *J Neuroendocrinol*, 20 Suppl 1, 124-9.

PAGOTTO, U., MARSICANO, G., COTA, D., LUTZ, B. & PASQUALI, R. (2006). The emerging role of the endocannabinoid system in endocrine regulation and energy balance. *Endocr Rev*, 27, 73-100.

PAJVANI, U. B., DU, X., COMBS, T. P., BERG, A. H., RAJALA, M. W., SCHULTHESS, T., ENGEL, J., BROWNLEE, M. & SCHERER, P. E. (2003). Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications for metabolic regulation and bioactivity. *J Biol Chem*, 278, 9073-85.

PATAKY, Z., BOBBIONI-HARSCH, E. & GOLAY, A. (2010). Open questions about metabolically normal obesity. *Int J Obes (Lond)*, 34 Suppl 2, S18-23.

PATEL, S., WOHLFEIL, E. R., RADEMACHER, D. J., CARRIER, E. J., PERRY, L. J., KUNDU, A., FALCK, J. R., NITHIPATIKOM, K., CAMPBELL, W. B. &

HILLARD, C. J. (2003). The general anesthetic propofol increases brain N-arachidonylethanolamine (anandamide) content and inhibits fatty acid amide hydrolase. *Br J Pharmacol*, 139, 1005-13.

PAVON, F. J., BILBAO, A., HERNANDEZ-FOLGADO, L., CIPPITELLI, A., JAGEROVIC, N., ABELLAN, G., RODRIGUEZ-FRANCO, M. A., SERRANO, A., MACIAS, M., GOMEZ, R., NAVARRO, M., GOYA, P. & RODRIGUEZ DE FONSECA, F. (2006). Antiobesity effects of the novel in vivo neutral cannabinoid receptor antagonist 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-hexyl-1H-1,2,4-triazole--LH 21. *Neuropharmacology*, 51, 358-66.

PAVON, F. J., SERRANO, A., PEREZ-VALERO, V., JAGEROVIC, N., HERNANDEZ-FOLGADO, L., BERMUDEZ-SILVA, F. J., MACIAS, M., GOYA, P. & DE FONSECA, F. R. (2008). Central versus peripheral antagonism of cannabinoid CB1 receptor in obesity: effects of LH-21, a peripherally acting neutral cannabinoid receptor antagonist, in Zucker rats. *J Neuroendocrinol*, 20 Suppl 1, 116-23.

PEETERS, A., BARENDREGT, J. J., WILLEKENS, F., MACKENBACH, J. P., AL MAMUN, A. & BONNEUX, L. (2003). Obesity in adulthood and its consequences for life expectancy: a life-table analysis. *Ann Intern Med*, 138, 24-32.

PERTWEE, R. G. (2006). The pharmacology of cannabinoid receptors and their ligands: an overview. *Int J Obes (Lond)*, 30 Suppl 1, S13-8.

PERTWEE, R. G., HOWLETT, A. C., ABOOD, M. E., ALEXANDER, S. P., DI MARZO, V., ELPHICK, M. R., GREASLEY, P. J., HANSEN, H. S., KUNOS, G., MACKIE, K., MECHOULAM, R. & ROSS, R. A. (2010). International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB₁ and CB₂. *Pharmacol Rev*, 62, 588-631.

PERTWEE, R. G. & ROSS, R. A. (2002). Cannabinoid receptors and their ligands. *Prostaglandins Leukot Essent Fatty Acids*, 66, 101-21.

PETITET, F., JEANTAUD, B., CAPET, M. & DOBLE, A. (1997). Interaction of brain cannabinoid receptors with guanine nucleotide binding protein: a radioligand binding study. *Biochem Pharmacol*, 54, 1267-70.

PI-SUNYER, F. X., ARONNE, L. J., HESHMATI, H. M., DEVIN, J. & ROSENSTOCK, J. (2006). Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: a randomized controlled trial. *JAMA*, 295, 761-75.

PIESTRZENIEWICZ, K., LUCZAK, K., KOMOROWSKI, J., MACIEJEWSKI, M., JANKIEWICZ WIKI, J. & GOCH, J. H. (2008). Resistin increases with obesity and atherosclerotic risk factors in patients with myocardial infarction. *Metabolism*, 57, 488-93.

PIOMELLI, D., TARZIA, G., DURANTI, A., TONTINI, A., MOR, M., COMPTON, T. R., DASSE, O., MONAGHAN, E. P., PARROTT, J. A. & PUTMAN, D. (2006). Pharmacological profile of the selective FAAH inhibitor KDS-4103 (URB597). *CNS Drug Rev*, 12, 21-38.

PORTER, A. C., SAUER, J. M., KNIERMAN, M. D., BECKER, G. W., BERNA, M. J., BAO, J., NOMIKOS, G. G., CARTER, P., BYMASTER, F. P., LEESE, A. B. & FELDER, C. C. (2002). Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor. *J Pharmacol Exp Ther*, 301, 1020-4.

RAKHSHAN, F., DAY, T., BLAKELY, R. & BARKER, E. (2000). Carrier-Mediated Uptake of the Endogenous Cannabinoid Anandamide in RBL-2H3 Cells. *J Pharmacol Exp Ther*, 292, 960-967.

REUL, B. A., ONGEMBA, L. N., POTTIER, A. M., HENQUIN, J. C. & BRICHARD, S. M. (1997). Insulin and insulin-like growth factor 1 antagonize the stimulation of ob gene expression by dexamethasone in cultured rat adipose tissue. *Biochem J*, 324 (Pt 2), 605-10.

ROBINSON, K., PRINS, J. & VENKATESH, B. (2011). Clinical review: Adiponectin biology and its role in inflammation and critical illness. *Crit Care*, 15, 221.

ROCHE, R., HOAREAU, L., BES-HOUTMANN, S., GONTHIER, M. P., LABORDE, C., BARON, J. F., HAFFAF, Y., CESARI, M. & FESTY, F. (2006). Presence of the cannabinoid receptors, CB1 and CB2, in human omental and subcutaneous adipocytes. *Histochem Cell Biol*, 126, 177-87.

ROCKWELL, C. E., SNIDER, N. T., THOMPSON, J. T., VANDEN HEUVEL, J. P. & KAMINSKI, N. E. (2006). Interleukin-2 suppression by 2-arachidonyl glycerol is mediated through peroxisome proliferator-activated receptor gamma independently of cannabinoid receptors 1 and 2. *Mol Pharmacol*, 70, 101-11.

RODBELL, M. (1964). Metabolism of Isolated Fat Cells. *J Biol Chem*, 239, 375-380.

RONTI, T., LUPATTELLI, G. & MANNARINO, E. (2006). The endocrine function of adipose tissue: an update. *Clin Endocrinol (Oxf)*, 64, 355-65.

ROSENSTOCK, J., HOLLANDER, P., CHEVALIER, S. & IRANMANESH, A. (2008). SERENADE: the Study Evaluating Rimnabant Efficacy in Drug-naive Diabetic Patients: effects of monotherapy with rimnabant, the first selective CB1 receptor antagonist, on glycemic control, body weight, and lipid profile in drug-naive type 2 diabetes. *Diabetes Care*, 31, 2169-76.

ROSS, R. A., CRAIB, S. J., STEVENSON, L. A., PERTWEE, R. G., HENDERSON, A., TOOLE, J. & ELLINGTON, H. C. (2002). Pharmacological characterization of the anandamide cyclooxygenase metabolite: prostaglandin E2 ethanolamide. *J Pharmacol Exp Ther*, 301, 900-7.

RYBERG, E., LARSSON, N., SJOGREN, S., HJORTH, S., HERMANSSON, N. O., LEONOVA, J., ELEBRING, T., NILSSON, K., DRMOTA, T. & GREASLEY, P. J. (2007). The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol*, 152, 1092-101.

SANDBERG, A. & FOWLER, C. (2005). Measurement of saturable and non-saturable components of anandamide uptake into P19 embryonic carcinoma cells in the presence of fatty acid-free bovine serum albumin. *Chem Phys Lipids*, 134, 131-139.

SARZANI, R., BORDICCHIA, M., MARCUCCI, P., BEDETTA, S., SANTINI, S., GIOVAGNOLI, A., SCAPPINI, L., MINARDI, D., MUZZONIGRO, G., DESSI-FULGHERI, P. & RAPPELLI, A. (2009). Altered pattern of cannabinoid type 1 receptor expression in adipose tissue of dysmetabolic and overweight patients. *Metabolism*, 58, 361-7.

SAVAGE, D. B., SEWTER, C. P., KLENK, E. S., SEGAL, D. G., VIDAL-PUIG, A., CONSIDINE, R. V. & O'RAHILLY, S. (2001). Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. *Diabetes*, 50, 2199-202.

SAVINAINEN, J. R., SAARIO, S. M., NIEMI, R., JARVINEN, T. & LAITINEN, J. T. (2003). An optimized approach to study endocannabinoid signaling: evidence against constitutive activity of rat brain adenosine A1 and cannabinoid CB1 receptors. *Br J Pharmacol*, 140, 1451-9.

SCHEEN, A. J., FINER, N., HOLLANDER, P., JENSEN, M. D. & VAN GAAL, L. F. (2006). Efficacy and tolerability of rimonabant in overweight or obese patients with type 2 diabetes: a randomised controlled study. *Lancet*, 368, 1660-72.

SCHERER, T. & BUETTNER, C. (2011). Yin and Yang of hypothalamic insulin and leptin signaling in regulating white adipose tissue metabolism. *Rev Endocr Metab Disord*.

SCHLOSBERG, J. E., BLANKMAN, J. L., LONG, J. Z., NOMURA, D. K., PAN, B., KINSEY, S. G., NGUYEN, P. T., RAMESH, D., BOOKER, L., BURSTON, J. J., THOMAS, E. A., SELLEY, D. E., SIM-SELLEY, L. J., LIU, Q. S., LICHTMAN, A. H. & CRAVATT, B. F. (2010). Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat Neuro*, 13, 1113-U111.

SCHMID, P. C., ZUZARTE-AUGUSTIN, M. L. & SCHMID, H. H. (1985). Properties of rat liver N-acylethanolamine amidohydrolase. *J Biol Chem*, 260, 14145-9.

SCHWARTZ, D. R. & LAZAR, M. A. (2011). Human resistin: found in translation from mouse to man. *Trends Endocrinol Metab*.

SETHI, J. K. & VIDAL-PUIG, A. J. (2007). Thematic review series: adipocyte biology. Adipose tissue function and plasticity orchestrate nutritional adaptation. *J Lipid Res*, 48, 1253-62.

SHOJIMA, N., SAKODA, H., OGIHARA, T., FUJISHIRO, M., KATAGIRI, H., ANAI, M., ONISHI, Y., ONO, H., INUKAI, K., ABE, M., FUKUSHIMA, Y., KIKUCHI, M., OKA, Y. & ASANO, T. (2002). Humoral regulation of resistin expression in 3T3-L1 and mouse adipose cells. *Diabetes*, 51, 1737-44.

SILVESTRI, C., LIGRESTI, A. & DI MARZO, V. (2011). Peripheral effects of the endocannabinoid system in energy homeostasis: adipose tissue, liver and skeletal muscle. *Rev Endocr Metab Disord*, 12, 153-62.

SIM, L. J., SELLEY, D. E. & CHILDERS, S. R. (1995). In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5'-[gamma-[35S]thio]-triphosphate binding. *Proc Natl Acad Sci U S A*, 92, 7242-6.

SIPE, J. C., WAALEN, J., GERBER, A. & BEUTLER, E. (2005). Overweight and obesity associated with a missense polymorphism in fatty acid amide hydrolase (FAAH). *Int J Obes (Lond)*, 29, 755-9.

SIRI, W. E. (ed.) 1961. *Techniques for measuring body composition: Body composition from fluid space and density*: Washington, DC: National Academy of Science.

SMART, D., JONSSON, K. O., VANDEVOORDE, S., LAMBERT, D. M. & FOWLER, C. J. (2002). 'Entourage' effects of N-acyl ethanolamines at human vanilloid receptors. Comparison of effects upon anandamide-induced vanilloid receptor activation and upon anandamide metabolism. *Br J Pharmacol*, 136, 452-8.

SON, M. H., KIM, H. D., CHAE, Y. N., KIM, M. K., SHIN, C. Y., AHN, G. J., CHOI, S. H., YANG, E. K., PARK, K. J., CHAE, H. W., MOON, H. S., KIM, S. H., SHIN, Y. G. & YOON, S. H. (2010). Peripherally acting CB1-receptor antagonist: the relative importance of central and peripheral CB1 receptors in adiposity control. *Int J Obes (Lond)*, 34, 547-56.

SPOTO, B., FEZZA, F., PARLONGO, G., BATTISTA, N., SGRO', E., GASPERI, V., ZOCCALI, C. & MACCARRONE, M. (2006). Human adipose tissue binds and metabolizes the endocannabinoids anandamide and 2-arachidonoylglycerol. *Biochimie*, 88, 1889-1897.

SPRANGER, J., KROKE, A., MÖHLIG, M., BERGMANN, M., RISTOW, M., BOEING, H. & PFEIFFER, A. (2003). Adiponectin and protection against type 2 diabetes mellitus. *The Lancet*, 361, 226-228.

STAHL, A., EVANS, J. G., PATEL, S., HIRSCH, D. & LODISH, H. F. (2002). Insulin causes fatty acid transport protein translocation and enhanced fatty acid uptake in adipocytes. *Dev Cell*, 2, 477-88.

STAROWICZ, K., CRISTINO, L., MATIAS, I., CAPASSO, R., RACIOPPI, A., IZZO, A. & DI MARZO, V. (2008). Endocannabinoid Dysregulation in the Pancreas and Adipose Tissue of Mice Fed With a High-fat Diet. *Obesity*, 16, 553-565.

STEPPAN, C. M., BAILEY, S. T., BHAT, S., BROWN, E. J., BANERJEE, R. R., WRIGHT, C. M., PATEL, H. R., AHIMA, R. S. & LAZAR, M. A. (2001). The hormone resistin links obesity to diabetes. *Nature*, 409, 307-12.

STIENSTRA, R., DUVAL, C., MULLER, M. & KERSTEN, S. (2007). PPARs, Obesity, and Inflammation. *PPAR Res*, 2007, 95974.

STOFKOVA, A. (2010). Resistin and visfatin: regulators of insulin sensitivity, inflammation and immunity. *Endocr Regul*, 44, 25-36.

STUMP, D. D., FAN, X. & BERK, P. D. (2001). Oleic acid uptake and binding by rat adipocytes define dual pathways for cellular fatty acid uptake. *J Lipid Res*, 42, 509-20.

SUGIURA, T., KONDO, S., SUKAGAWA, A., NAKANE, S., SHINODA, A., ITOH, K., YAMASHITA, A. & WAKU, K. (1995). 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun*, 215, 89-97.

SUN, Y., ALEXANDER, S. P., KENDALL, D. A. & BENNETT, A. J. (2006). Cannabinoids and PPAR α signalling. *Biochem Soc Trans*, 34, 1095-7.

SUN, Y. X., TSUBOI, K., OKAMOTO, Y., TONAI, T., MURAKAMI, M., KUDO, I. & UEDA, N. (2004). Biosynthesis of anandamide and N-palmitoylethanolamine by sequential actions of phospholipase A2 and lysophospholipase D. *Biochem J*, 380, 749-56.

SUN, Y. X., TSUBOI, K., ZHAO, L. Y., OKAMOTO, Y., LAMBERT, D. M. & UEDA, N. (2005). Involvement of N-acylethanolamine-hydrolyzing acid amidase in the degradation of anandamide and other N-acylethanolamines in macrophages. *Biochim Biophys Acta*, 1736, 211-20.

TAKSALI, S., CAPRIO, S., DZIURA, J., DUFOUR, S., CALÍ, A., GOODMAN, R., PAPADEMETRIS, X., BURGERT, T., PIERPONT, B., SAVOYE, M., SHAW, M., SEYAL, A. & WEISS, R. (2008). High Visceral and Low Abdominal Subcutaneous Fat Stores in the Obese Adolescent: A Determinant of an Adverse Metabolic Phenotype *Diabetes*, 57, 367-371.

TAM, J., LIU, J., MUKHOPADHYAY, B., CINAR, R., GODLEWSKI, G. & KUNOS, G. (2011). Endocannabinoids in liver disease. *Hepatology*, 53, 346-55.

TAM, J., VEMURI, V. K., LIU, J., BATKAI, S., MUKHOPADHYAY, B., GODLEWSKI, G., OSEI-HYIAMAN, D., OHNUMA, S., AMBUDKAR, S. V., PICKEL, J., MAKRIYANNIS, A. & KUNOS, G. (2010). Peripheral CB1 cannabinoid receptor blockade improves cardiometabolic risk in mouse models of obesity. *J Clin Invest*, 120, 2953-66.

TEIXEIRA, D., PESTANA, D., FARIA, A., CALHAU, C., AZEVEDO, I. & MONTEIRO, R. (2010). Modulation of adipocyte biology by delta(9)-tetrahydrocannabinol. *Obesity (Silver Spring)*, 18, 2077-85.

THORS, L., ERIKSSON, J. & FOWLER, C. (2007). Inhibition of the cellular uptake of anandamide by genistein and its analogue daidzein in cells with different levels of fatty acid amide hydrolase-driven uptake. *Br J Pharmacol*, 152, 744-750.

TOMINAGA, M. & CATERINA, M. J. (2004). Thermosensation and pain. *J Neurobiol*, 61, 3-12.

TOPOL, E. J., BOUSSER, M. G., FOX, K. A., CREAGER, M. A., DESPRES, J. P., EASTON, J. D., HAMM, C. W., MONTALESCOT, G., STEG, P. G., PEARSON, T. A., COHEN, E., GAUDIN, C., JOB, B., MURPHY, J. H. & BHATT, D. L. (2010). Rimonabant for prevention of cardiovascular events (CRESCENDO): a randomised, multicentre, placebo-controlled trial. *Lancet*, 376, 517-23.

TORNQVIST, H. & BELFRAGE, P. (1976). Purification and some properties of a monoacylglycerol-hydrolyzing enzyme of rat adipose tissue. *J Biol Chem*, 251, 813-9.

TORNQVIST, H., NILSSON-EHLE, P. & BELFRAGE, P. (1978). Enzymes catalyzing the hydrolysis of long-chain monoacylglycerols in rat adipose tissue. *Biochim Biophys Acta*, 530, 474-86.

TOURINO, C., OVEISI, F., LOCKNEY, J., PIOMELLI, D. & MALDONADO, R. (2010). FAAH deficiency promotes energy storage and enhances the motivation for food. *Int J Obes (Lond)*, 34, 557-68.

TRAYHURN, P., BING, C. & WOOD, I. S. (2006). Adipose tissue and adipokines--energy regulation from the human perspective. *J Nutr*, 136, 1935S-1939S.

UEDA, H., KOBAYASHI, T., KISHIMOTO, M., TSUTSUMI, T. & OKUYAMA, H. (1993). A possible pathway of phosphoinositide metabolism through EDTA-insensitive phospholipase A1 followed by lysophosphoinositide-specific phospholipase C in rat brain. *J Neurochem*, 61, 1874-81.

UEDA, N., KURAHASHI, Y., YAMAMOTO, S. & TOKUNAGA, T. (1995a). Partial purification and characterization of the porcine brain enzyme hydrolyzing and synthesizing anandamide. *J Biol Chem*, 270, 23823-7.

UEDA, N., YAMAMOTO, K., YAMAMOTO, S., TOKUNAGA, T., SHIRAKAWA, E., SHINKAI, H., OGAWA, M., SATO, T., KUDO, I., INOUE, K. & ET AL. (1995b). Lipoxygenase-catalyzed oxygenation of arachidonylethanolamide, a cannabinoid receptor agonist. *Biochim Biophys Acta*, 1254, 127-34.

UNGER, R. (1997). How Obesity Causes Diabetes in Zucker Diabetic Fatty Rats. *Trends Endocrinol Metab*, 8, 276-282.

VALSAMAKIS, G., MCTERNAN, P. G., CHETTY, R., AL DAGHRI, N., FIELD, A., HANIF, W., BARNETT, A. H. & KUMAR, S. (2004). Modest weight loss and reduction in waist circumference after medical treatment are associated with favorable changes in serum adipocytokines. *Metabolism*, 53, 430-4.

VAN GAAL, L., PI-SUNYER, X., DESPRÉS, J., MCCARTHY, C. & SCHEEN, A. (2008a). Efficacy and safety of rimonabant for improvement of multiple cardiometabolic risk factors in overweight/obese patients: Pooled 1-year data from the rimonabant in obesity (RIO) program. *Diab Care*, 31, S229-S240.

VAN GAAL, L., RISSANEN, A., SCHEEN, A., ZIEGLER, O. & RÖSSNER, S. (2005). Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factor in overweight patients: 1-year experience from the RIO-Europe study. *The Lancet*, 365, 1389-1397.

VAN GAAL, L., SCHEEN, A., RISSANEN, A., RÖSSNER, S., HANOTIN, C., ZIEGLER, O. & GROUP, R.-E. S. (2008b). Long-term effect of CB1 blockade with rimonabant on cardiometabolic risk factors: two year results from the RIO-Europe Study. *European Heart Journal*, 29, 1761-1771.

VAN, R. L., BAYLISS, C. E. & RONCARI, D. A. (1976). Cytological and enzymological characterization of adult human adipocyte precursors in culture. *J Clin Invest*, 58, 699-704.

VARLAMOV, O., SOMWAR, R., CORNEA, A., KIEVIT, P., GROVE, K. L. & ROBERTS, C. T., JR. (2010). Single-cell analysis of insulin-regulated fatty acid uptake in adipocytes. *Am J Physiol Endocrinol Metab*, 299, E486-96.

VAUGHAN, M., BERGER, J. E. & STEINBERG, D. (1964). Hormone-Sensitive Lipase and Monoglyceride Lipase Activities in Adipose Tissue. *J Biol Chem*, 239, 401-9.

VETTOR, R. & PAGANO, C. (2009). The role of the endocannabinoid system in lipogenesis and fatty acid metabolism. *Best Pract Res Clin Endocrinol Metab*, 23, 51-63.

VILLENA, J. A., ROY, S., SARKADI-NAGY, E., KIM, K. H. & SUL, H. S. (2004). Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis. *J Biol Chem*, 279, 47066-75.

WADDEN, T. A., FUJIOKA, K., TOUBRO, S., GANTZ, I., ERONDU, N. E., CHEN, M., SURYAWANSHI, S., CAROFANO, W., JOHNSON-LEVONAS, A. O., SHAPIRO, D. R., KAUFMAN, K. D., HEYMSFIELD, S. B. & AMATRUDA, J. M. (2010). A randomized trial of lifestyle modification and taranabant for maintaining weight loss achieved with a low-calorie diet. *Obesity (Silver Spring)*, 18, 2301-10.

WAJCHENBERG, B. L. (2000). Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocr Rev*, 21, 697-738.

WANG, J., OKAMOTO, Y., TSUBOI, K. & UEDA, N. (2008a). The stimulatory effect of phosphatidylethanolamine on N-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD). *Neuropharmacology*, 54, 8-15.

WANG, P., MARIMAN, E., RENES, J. & KEIJER, J. (2008b). The secretory function of adipocytes in the physiology of white adipose tissue. *J Cell Physiol*, 216, 3-13.

WATTS, G. F. & CHAN, D. C. (2010). Mechanisms for therapeutic correction of dyslipidaemia in insulin resistance and diabetes. *Atheroscler Suppl*, 11, 61-4.

WEI, B. Q., MIKKELSEN, T. S., MCKINNEY, M. K., LANDER, E. S. & CRAVATT, B. F. (2006). A second fatty acid amide hydrolase with variable distribution among placental mammals. *J Biol Chem*, 281, 36569-78.

WEISBERG, S. P., MCCANN, D., DESAI, M., ROSENBAUM, M., LEIBEL, R. L. & FERRANTE, A. W., JR. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest*, 112, 1796-808.

WHITLOCK, G., LEWINGTON, S., SHERLIKER, P., CLARKE, R., EMBERSON, J., HALSEY, J., QIZILBASH, N., COLLINS, R. & PETO, R. (2009). Body-mass index and cause-specific mortality in 900 000 adults: collaborative analyses of 57 prospective studies. *Lancet*, 373, 1083-96.

WHO (2006a). Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia. Report of a WHO/IDF consultation. <http://www.who.int/diabetes/en/>.

WHO (2006b). Obesity and overweight: factsheet 311. <http://www.who.int/topics/obesity/en/>.

WILD, S., ROGLIC, G., GREEN, A., SICREE, R. & KING, H. (2004). Global Prevalence of Diabetes - Estimates for the year 2000 and projections for 2030. *Diabetes Care*, 27, 1047-1053.

WILLIAMS, G. & FRÜHBECK, G. (eds.) 2009. *Obesity: Science to Practice*: Wiley-Blackwell.

WILSON, S. J., LOVENBERG, T. W. & BARBIER, A. J. (2003). A high-throughput-compatible assay for determining the activity of fatty acid amide hydrolase. *Anal Biochem*, 318, 270-5.

XIANG, C., WU, Y., MA, L., DING, L., LISINSKI, I., BROWNSTEIN, M., CUSHMAN, S. & CHEN, X. (2007). Characterisation of insulin-resistant phenotype of cultured rat primary adipose cells. *Diabetologia*, 50, 1070-1079.

XIONG, W., HOSOI, M., KOO, B. N. & ZHANG, L. (2008). Anandamide inhibition of 5-HT_{3A} receptors varies with receptor density and desensitization. *Mol Pharmacol*, 73, 314-22.

YAMAUCHI, T., NIO, Y., MAKI, T., KOBAYASHI, M., TAKAZAWA, T., IWABU, M., OKADA-IWABU, M., KAWAMOTO, S., KUBOTA, N., KUBOTA, T., ITO, Y., KAMON, J., TSUCHIDA, A., KUMAGAI, K., KOZONO, H., HADA, Y., OGATA, H., TOKUYAMA, K., TSUNODA, M., IDE, T., MURAKAMI, K., AWAZAWA, M., TAKAMOTO, I., FROGUEL, P., HARA, K., TOBE, K., NAGAI, R., UEKI, K. & KADOWAKI, T. (2007). Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. *Nat Med*, 13, 332-9.

YATES, M. & BARKER, E. (2009a). Inactivation and Biotransformation of the Endogenous Cannabinoids Anandamide and 2-Arachidonoylglycerol. *Mol Pharmacol*, 76, 11-17.

YATES, M. L. & BARKER, E. L. (2009b). Organized trafficking of anandamide and related lipids. *Vitam Horm*, 81, 25-53.

YILDIZ, B. O., SUCHARD, M. A., WONG, M. L., MCCANN, S. M. & LICINIO, J. (2004). Alterations in the dynamics of circulating ghrelin, adiponectin, and leptin in human obesity. *Proc Natl Acad Sci U S A*, 101, 10434-9.

YOO, W., LEE, J., PARK, S., KIM, Y. S., LIM, C., YOON, E., HUR, G. & OH, J. (2010). Albumin expression is required for adipocyte differentiation of 3T3-L1 cells. *Biochem Biophys Res Commun*, 397, 170-5.

ZECHNER, R., KIENESBERGER, P. C., HAEMMERLE, G., ZIMMERMANN, R. & LASS, A. (2009). Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores. *J Lipid Res*, 50, 3-21.

ZHANG, L. L., YAN LIU, D., MA, L. Q., LUO, Z. D., CAO, T. B., ZHONG, J., YAN, Z. C., WANG, L. J., ZHAO, Z. G., ZHU, S. J., SCHRADER, M., THILO, F., ZHU, Z. M. & TEPEL, M. (2007). Activation of transient receptor potential vanilloid type-1 channel prevents adipogenesis and obesity. *Circ Res*, 100, 1063-70.

ZHANG, Y., PROENCA, R., MAFFEI, M., BARONE, M., LEOPOLD, L. & FRIEDMAN, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature*, 372, 425-32.

ZIMMERMANN, R., STRAUSS, J. G., HAEMMERLE, G., SCHOISWOHL, G., BIRNER-GRUENBERGER, R., RIEDERER, M., LASS, A., NEUBERGER, G., EISENHABER, F., HERMETTER, A. & ZECHNER, R. (2004). Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science*, 306, 1383-6.

ZWEYTICK, D., ATHENSTAEDT, K. & DAUM, G. (2000). Intracellular lipid particles of eukaryotic cells. *Biochim Biophys Acta*, 1469, 101-20.

ZYGMUNT, P. M., PETERSSON, J., ANDERSSON, D. A., CHUANG, H., SORGARD, M., DI MARZO, V., JULIUS, D. & HOGESTATT, E. D. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature*, 400, 452-7.